STUDIES ON THE CHEMICAL AND BIOLOGICAL PROPERTIES OF ANTITUMOUR TRIAZENES

by

JULIE KAY HORTON

A thesis submitted for the degree of

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Chemical and biological properties of 1-aryl-3,3-dialkyltriazenes were studied with the aim of elucidating their mode of action and the identity of the active antitumour species.

Photolysis of DTIC at alkaline pH afforded 2-azahypoxanthine, and at mildly acidic pH (2 - 6) 4-carbomoylimidazolium-5-olate. 5-Diazoimidazole-4-carboxamide, an intermediate in the photodecomposition of DTIC, was responsible for the interaction of DTIC with nucleophiles such as thiol compounds in the presence of sunlight.

Another dimethyltriazene, 1-(4-acetylphenyl)-3,3-dimethyltriazene (AcDMT) was photostable, but subject to protolysis at pH $<$ 5.2. The stability of monomethyl- but not hydroxymethyl- triazenes (both dimethyltriazene metabolites) at physiological pH, measured by UV spectroscopy, appeared great enough to allow distribution in vivo to a distant tumour site.

Certain properties of N-demethylation of AcDMT such as species differences, investigated by normal phase HPLC, differed from the N-demethylation of aminopyrine, a model substrate of cytochrome P-450 N-demethylation. The metabolism of dialkyltriazenes under conditions of the bioassay was studied by reverse phase HPLC and monomethyltriazenes were identified as metabolic products of active antitumour derivatives. Monoalkyltriazenes were themselves metabolised, probably yielding a product other than the corresponding arylamine.

1-(4-Acetylphenyl)-3-methyltriazene (AcMMT) was significantly less toxic to hepatocytes than AcDMT when cell viability was assayed by the trypan blue dye exclusion test. Incubation of TLX5 lymphoma cells with AcMMT resulted in a significantly greater reduction in viability of the triazene sensitive than the resistant cell lines. Neither TLX5 cell line was able to activate AcDMT, or to metabolise AcMMT. There was no significant difference between the two TLX5 cell lines in their total glutathione content.

Monoalkyltriazenes, but not arylamines, prevented the oxidation of glutathione in mouse liver homogenate in Earl's buffer. AcMMT depleted total glutathione levels in mouse hepatocytes, and the increase in extracellular total glutathione could not account for intracellular loss. AcMMT and AcDMT did not inhibit the enzyme glutathione reductase.

Key words: DTIC photodecomposition, aryltriazene N-dealkylation, antitumour species, triazene resistance, glutathione.

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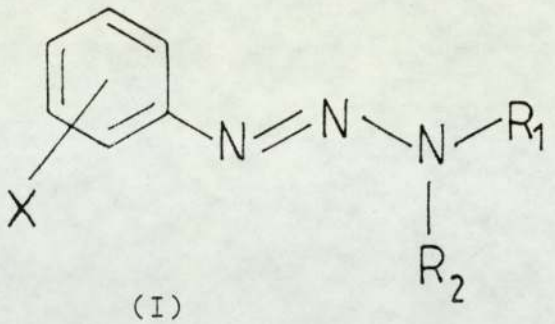
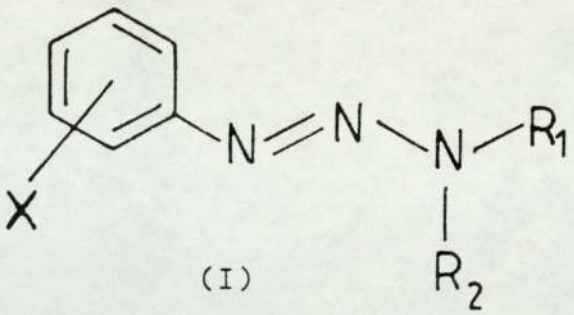
ABBREVIATIONS

HPLC	High Performance Liquid Chromatography
i.p.	Intra-peritoneal
tlc	Thin Layer Chromatography
UV	Ultra-violet
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BSA	Bovine serum albumin
DMSO	Dimethylsulphoxide
DTNB	5,5′-dithiobis(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis(β-aminoethyl ether) N,N′-tetraacetic acid
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
GSH	Glutathione (reduced form)
GSSG	Glutathione (oxidised form)
HEPES	N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid
NADH	β-Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NEDA	N-(Naphth-1-yl)ethylenediamine
TCA	Trichloroacetic acid
Tris	Tris(hydroxymethyl)aminomethane



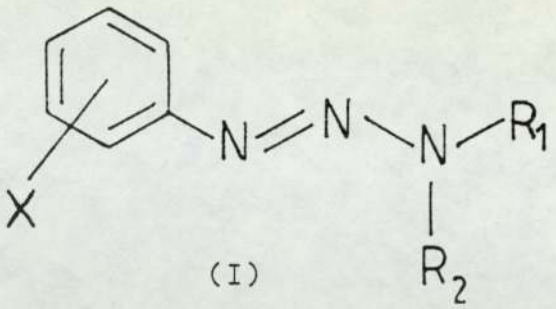
(I)

	X	R_1	R_2
(Ia)	H	Me	Me
(Ib)	H	Me	H
(Ic)	H	Et	Et
(Id)	H	Me	Ac
(Ie)	R	Me	CH_2OH
(Iee)	R	CH_2OH	H
(If)	4-CN	Et	CH_2OH
(Ig)	4-$CONH_2$	Me	H
(Ih)	4-$CONH_2$	Me	Me
(Ii)	4-COOH	Me	Me

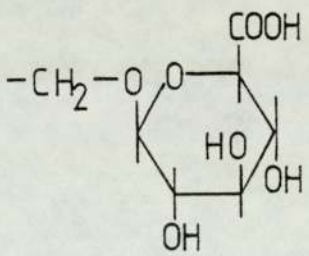


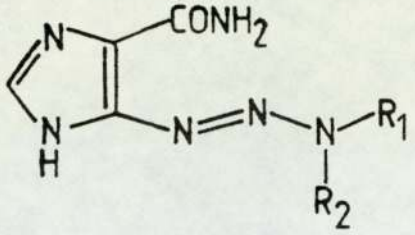
(I)

	X	R_1	R_2	
(Ij)	4-Ac	Me	Me	AcDMT
(Ik)	4-Ac	Me	H	AcMMT
(Il)	4-Ac	Me	CH_2OH	
(Im)	4-Ac	Et	Et	AcDET
(In)	4-Ac	Et	H	AcMET
(Io)	4-CH_3CHOH	Me	Me	
(Ip)	4-CH_3CHOH	Et	Et	
(Iq)	4-CN	Me	Me	CyDMT
(Ir)	4-CN	Me	H	CyMMT
(Is)	4-CN	Et	Et	CyDET
(It)	4-CN	Et	H	CyMET
(Iu)	4-CN	Me	Et	
(Iv)	4-CN	Me	t-butyl	
(Iw)	4-CN	t-butyl	H	



(I)

	X	R_1	R_2
(Ix)	4-CO_2Me	Me	Me
(Iy)	4-CO_2Me	Me	H
(Iz)	4-CO_2Me	Me	CH_2OH
(Iaa)	2,4,6-trichloro	Me	Me
(Ibb)	2,4,6-trichloro	Me	H
(Icc)	2,4,6-trichloro	Me	
(Idd)	4-NO_2	Me	H



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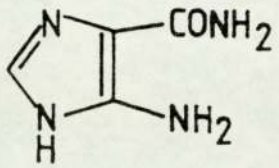
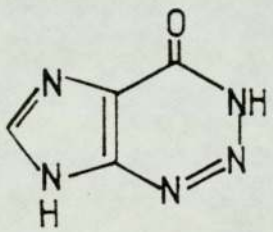


(III)

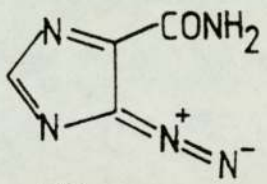
AIC

	R_1	R_2	
(IIa)	Me	Me	DTIC
(IIb)	Me	H	MTIC
(IIc)	CH_2CH_2Cl	CH_2CH_2Cl	BIC
(IId)	CH_3	CH_2OH	



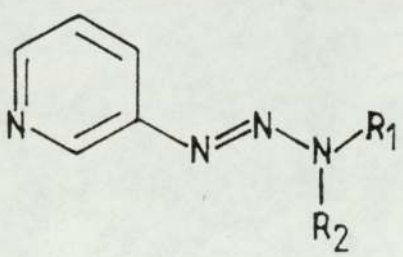
(IV)

2AH

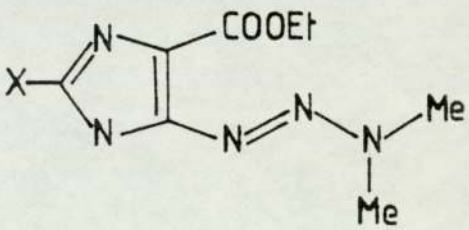


(V)

diazo-IC



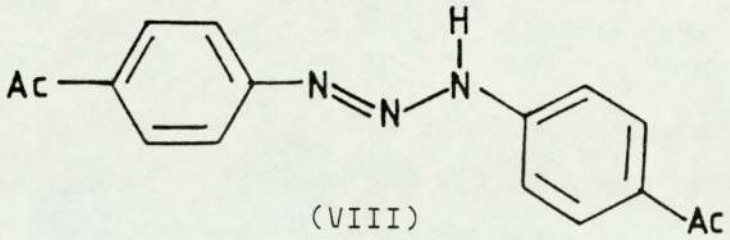
(VI)



(VII)

	R_1	R_2
(VIa)	Me	Me
(VIb)	Et	Et

	X
(VIIa)	Ph
(VIIb)	Me



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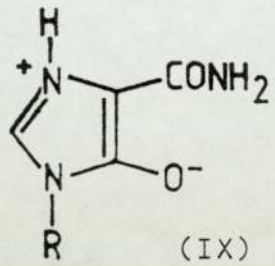
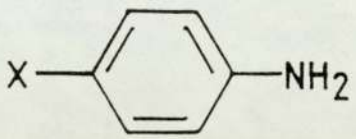
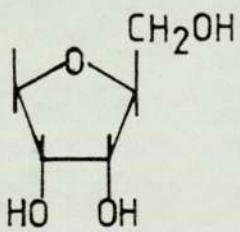
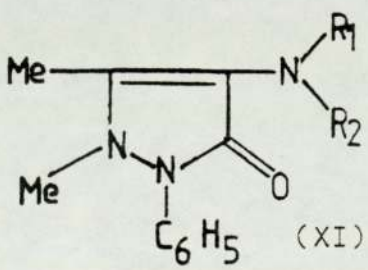


(IX)



(X)

	R		X	
(IXa)	H	OIC	(Xa)	Ac
			(Xb)	CN
(IXb)			(Xc)	CO_2Me



(XI)

	R_1	R_2	
(XIa)	Me	Me	aminopyrine
(XIb)	Me	H	
(XIc)	H	H	
(XId)	CHO	H	
(XIe)	Et	Et	
(XIf)	Et	H	
(XIg)	Ac	H	

PART A

INTRODUCTION

PART A INTRODUCTION

A.1 History and Development of Triazenes

A.1.1 Discovery of antitumour activity

The tumour inhibitory activity of a triazene, 1-phenyl-3,3-dimethyltriazene (Ia) was first observed by Clarke *et al* in 1955, who measured the *in vivo* growth-inhibition of a murine tumour, the Sarcoma 180 (1). Triazene antitumour activity was also observed against various strains of mouse leukemia and the relative inactivity of a diethyltriazene (Ic) was noted (2). Screening of structural variations of (Ia) indicated the importance of a 3-methyl group for the antitumour activity, whereas the structural features of the 1-aryl group were less crucial (3).

A.1.2 Events leading to the synthesis of DTIC (IIa)

A research programme designed to discover new inhibitors of nucleic acid biosynthesis led to the development of the triazene series (4). One of the intermediary products of *de novo* synthesis of purines is 5-aminoimidazole-4-carboxamide ribonucleotide (figure 1). It was suggested that derivatives of 5-aminoimidazole-4-carboxamide (AIC, III) may act as anti-metabolites, and possibly as *selective* agents since AIC had been found to be more rapidly utilized by tumour cells than normal tissues (5). In fact AIC was subsequently shown to stimulate tumour growth (6,7).

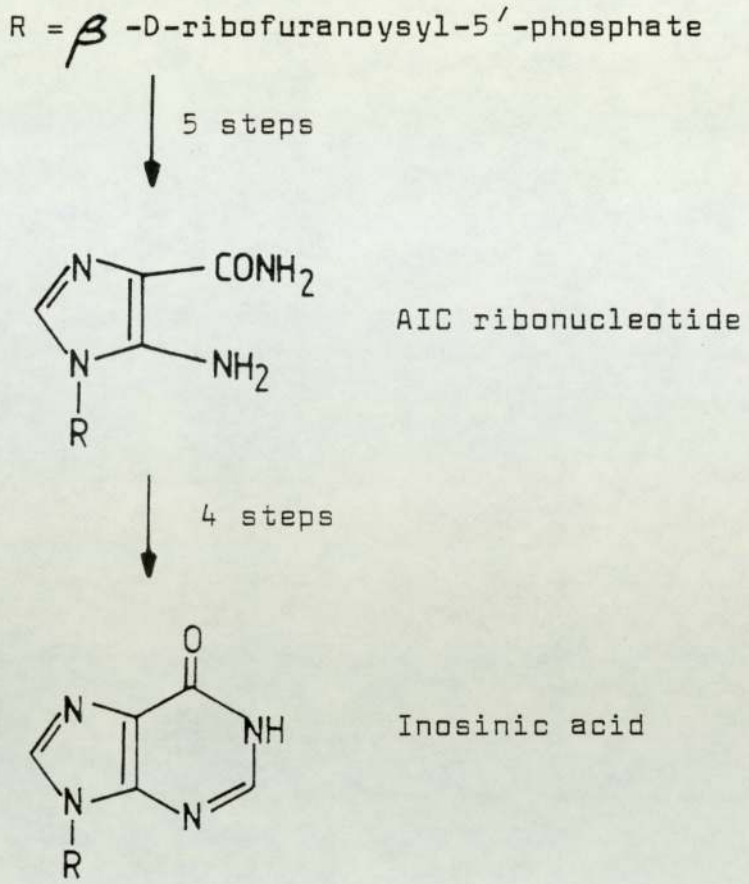


Figure 1 De novo biosynthesis of purine nucleotides

Treatment of AIC with nitrous acid had been reported to yield 2-azahypoxanthine (2AH, IV) (8), an inactive antibacterial agent. However, Shealy and co-workers now observed that the initial product of diazotisation of AIC was 5-diazoimidazole-4-carboxamide (diazo-IC, V), which then intramolecularly cyclizes to 2AH(9) (figure 2). Diazo-IC exhibits antitumour activity both in vitro and in vivo (9,10) whereas 2AH is inactive against the same murine tumour systems (9).

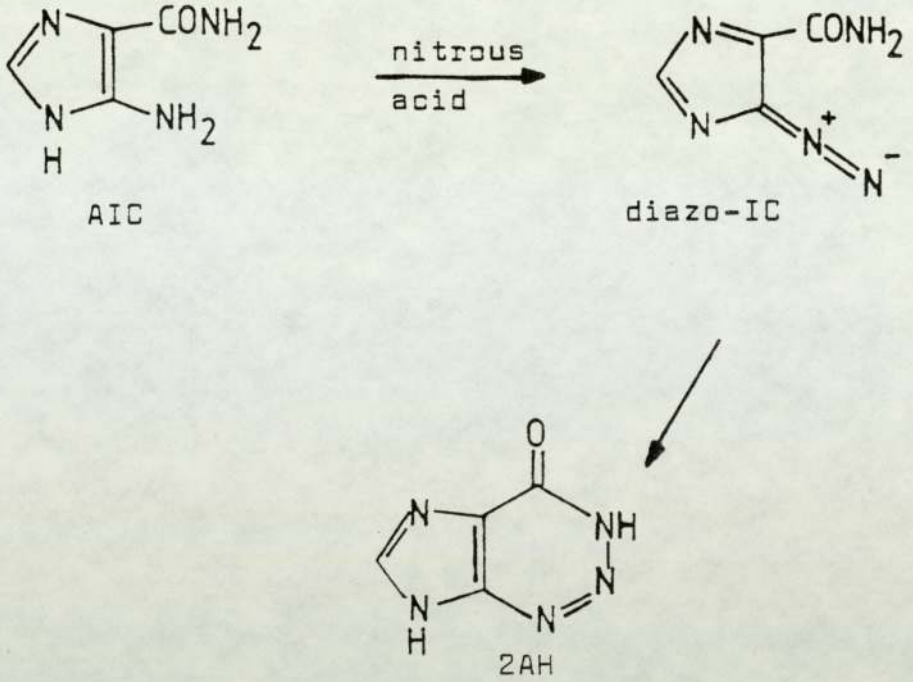


Figure 2 Diazotisatisation of AIC

The search for more stable and more active anti-tumour derivatives led to the synthesis of reaction products of diazo-IC with aliphatic amines - the imidazotriazenes - and most notably 5-(3,3-dimethyltriazen-1-yl)imidazole-4-carboxamide (DTIC, IIa) (11). The development of DTIC into a drug used in cancer chemotherapy has been summarized (12).

A.1.3 Triazene activity against experimental tumours

DTIC was found to be an active antitumour agent against L1210 leukemia, and a range of murine tumours including solid tumours and using a variety of dose regimes and combinations (4). 5-(3-Methyltriazen-1-yl)imidazole-4-carboxamide (MTIC, IIb), the desmethyl derivative of DTIC, also showed some activity but was difficult to evaluate because of its instability (13).

After these initial observations of antitumour activity, Shealy and co-workers synthesized a series of heteroaryl- and aryl- triazenes (11,14,15), and almost all were evaluated against L1210 leukemia. Again it was noted that, in general, activity was seen where there was present at least one 3-methyl group, but, for the phenyltriazenes in particular, activity was variable. Over the years, other workers have added to the vast array of triazenes which exhibit antitumour activity such as benzamide derivatives, substituted hydrazides and halogenated quinolines (16-19).

A.1.4 The clinical use of triazenes

DTIC has proved to be the only triazene with clinical usefulness. Another derivative, 5-[3,3-bis(2-chloroethyl)triazen-1-yl]-imidazole-4-carboxamide (BIC, IIc) was one of the most active agents against L1210 (20), but was disappointing in the clinic with frequent toxic side effects (21).

A phase I study of DTIC resulted in objective antitumour responses in patients with malignant melanoma and other disseminated diseases (22). Since then, the main clinical studies on DTIC have been against melanoma (23), a tumour very refractory to chemotherapy. A standard regime of 250 mg/m^2/day for 5 days with courses repeated at four week intervals has been adopted (24), with dose adjustment according to myelosuppression. The use of DTIC as a single agent in the treatment of melanoma has been reviewed (25,26), and the drug appears to be the most consistently active chemotherapeutic agent against the disease (25). However, despite a response rate of 20-30%, the complete remission rate is less

than 5% (27) and there is no real evidence for increased survival times.

Combinations of 2-5 drugs including DTIC have also been employed against melanoma (26,28). Many of these studies have not been carefully controlled, and there is no conclusive evidence that combination chemotherapy enhances response rate, duration of response or survival (29-32). DTIC has been used in chemoimmunotherapy regimes with varying results (33,34) and less frequently in the treatment of other cancers, such as a single agent against lymphoma (35) and in combination regimes against metastatic sarcomas (36) and Hodgkin's disease (37).

A.2 Metabolic Activation of Triazenes

A.2.1 In vivo disposition of DTIC

After administration to the dog and to man, DTIC was quickly cleared from the plasma (38). DTIC was detected in the urine together with AIC and an unknown metabolite with a triazene linkage (38-40). DTIC was presumed to undergo extensive degradation since no more than half the dose was excreted unchanged (40). Only when ^{14}C-methyl labelled DTIC was administered to mice was $^{14}CO_2$ detected in the expired air, and only after administration of ^{14}C-ring labelled drug was radioactive AIC found in the urine (40).

A.2.2 In vitro metabolism of phenyltriazenes

Preussmann et al showed that 1-phenyl-3,3-dimethyltriazene (Ia) was oxidatively N-demethylated by the microsomal fraction of rat liver in the presence of an NADPH generating system and oxygen, with the production of formaldehyde and aniline in roughly equimolar quantities (41) (figure 3). An intermediate of demethylation, phenylmethyltriazene (Ib), is a known alkylating agent and was considered to be the cytotoxic species generated from the dimethyltriazene (Ia). Other dimethyl- and diethyl- triazenes (Ic, VIa-b) were also shown to undergo dealkylation.

More recent results on the metabolism of both dimethyl- and monomethyl- phenyltriazenes will be discussed together with findings reported in this thesis.

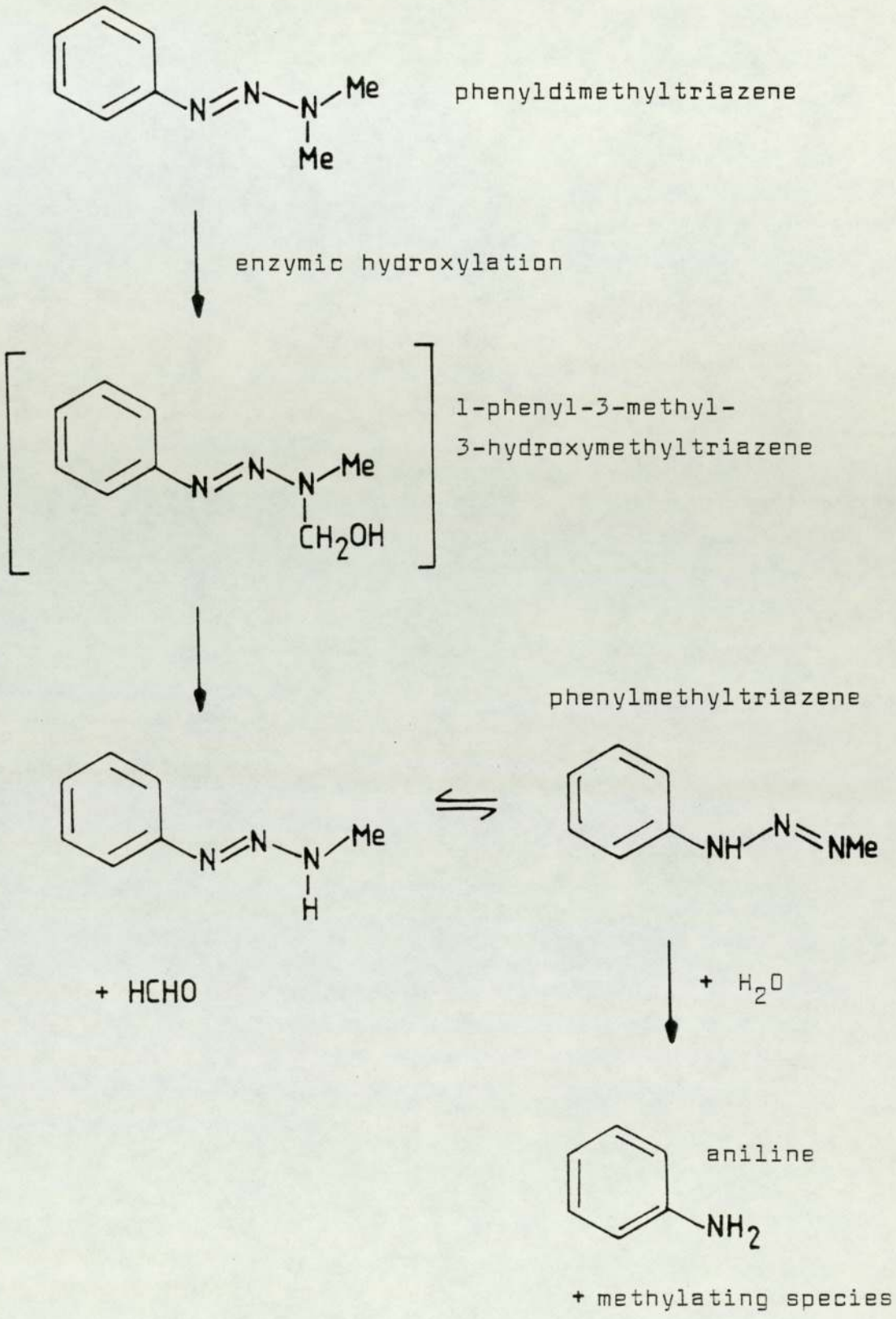


Figure 3 Proposed mechanism of activation for phenyldimethyltriazene.

A.2.3 Metabolism of DTIC

A similar N-demethylation process for DTIC would explain the early *in vivo* results (40). Further evidence for this pathway was that *in vitro* activation of DTIC resulted in the formation of formaldehyde and AIC and that pretreatment with phenobarbitone increased expiration of $^{14}CO_2$ after administration of ^{14}C-methyl labelled DTIC (42). Later *in vitro* metabolic studies again revealed AIC as a product of microsomal degradation of both DTIC and BIC (43).

A.2.4 Metabolism as a prerequisite for antitumour activity.

A.2.4.1 In vitro activity of phenyltriazenes

Phenyldimethyltriazene (Ia) was only weakly cytocidal against L1210 cells *in vitro* (44), and phenyldimethyltriazenes in general were totally inactive against TLX5 cells in the absence of a liver activating system (45-48). The monomethyl metabolite has been postulated as the active antitumour species since, in contrast, it exhibits antitumour activity *in vitro* without metabolic activation (44,45) and is also active *in vivo* (45,46).

A.2.4.2 Hydroxymethyltriazenes as the active antitumour species

Hydroxymethyltriazenes (Ie) have always been thought of as unstable metabolic intermediates until the recent identification of the hydroxymethyl metabolite of DTIC (IId) and of an O-glucuronide of an hydroxymethyltriazene (Icc) in rat urine after administration of the parent dimethyltriazenes (49,50). Hydroxymethyltriazenes are at least as active *in vivo*

against TLX5 lymphoma as the corresponding monomethyl- and dimethyl- triazene (46). However, a study both *in vitro* and *in vivo* using triazene sensitive and resistant TLX5 lines, suggested that the hydroxymethyl derivative was exerting its antitumour activity via its decomposition product, the monomethyltriazene (51). A 3-ethyl-3-hydroxymethyltriazene (If), which decomposes to yield the inactive monoethyltriazene, showed no antitumour activity *in vivo* (51). This lends further support to the hypothesis that the hydroxymethyl moiety *per se* is not the active species. Conjugation of the hydroxy group has been shown to markedly decrease *in vivo* activity, and the conjugate was not cytotoxic to TLX5 cells *in vitro* (52).

A.2.4.3 *N*-demethylation and antitumour activity

There is evidence that dimethyltriazenes must be metabolically activated (47), and yet early results revealed triazenes that are active antitumour agents but are not N-demethylated (41,53). Giraldi and co-workers too could not demonstrate a correlation between *in vitro* N-demethylation and *in vivo* antitumour activity (54). The work of Abel *et al* suggests that the *in vitro* activation of a dimethyltriazene (Ih) was not entirely due to N-demethylation, and that another metabolic route may be responsible (55).

The extent of N-demethylation of aryldimethyltriazenes has been shown to be dependent on ring substitution (56,57), whereas there is some evidence that antitumour activity is not influenced by the ring substituent (45). Derivatives with electron withdrawing substituents which stabilize triazenes are dealkylated to a greater extent than more labile triazene

derivatives (56); tri-substitution of the phenyl group blocks ring hydroxylation, also leading to preferential N-dealkylation (57). In contrast, Godin et al showed that formaldehyde liberation during metabolism of a series of aryldimethyltriazenes was not dependent on ring substitution (58). This result was in partial agreement with the work of Connors et al who found little variation in antitumour activity with a change in aryl substituent (45). However, the latter observation is still unexpected, since even if the monomethyltriazenes are liberated at the same rate, their stability and therefore ability to reach a tumour target site, should still be markedly influenced by the nature of the ring substituent.

A.2.4.4. Activation of DTIC

A.2.4.4.1 Photodecomposition pathway

The light-catalysed decomposition pathway for DTIC as first described by Shealy (11) (figure 4), is presumed to account for the in vitro activity of DTIC (59-61), but most reviewers agree there is little evidence for the rôle of these photoproducts in its in vivo antitumour activity (62,63). It has been suggested by other workers, however, that the photodecomposition pathway may be operative at all times, and even in vivo in the absence of light the generation of diazo-IC from DTIC never entirely ceases (64).

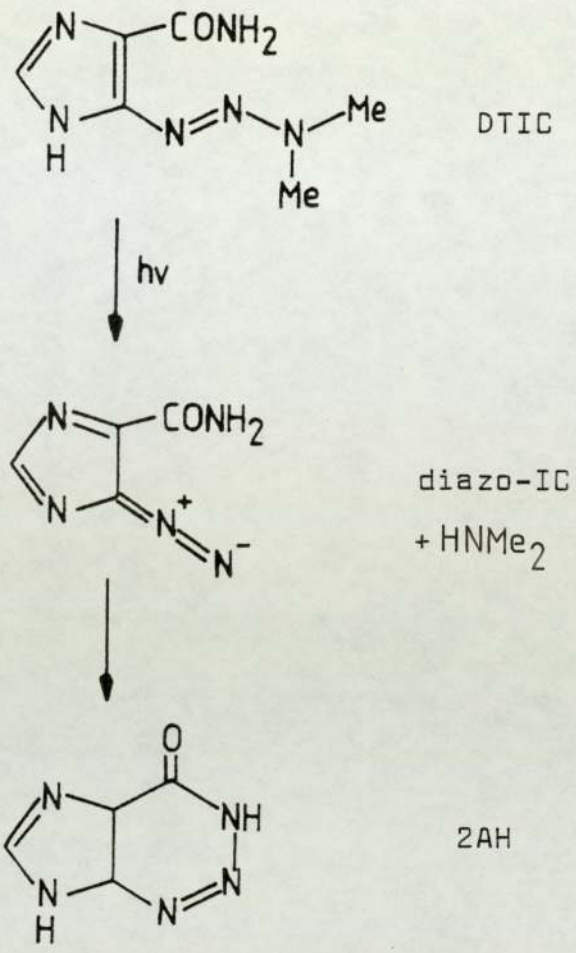


Figure 4 Photodecomposition pathway for DTIC

Table 1 summarises the <u>in vitro</u> activity of DTIC, which in general is more pronounced in the light. Diazo-IC and MTIC were equi-active in light and dark in many of the test systems.

Table 1 *In vitro* activity of DTIC

Test system	Effect of light on DTIC activity	Reference
B.subtilis	Increases	59
E.coli B	-	60
Chinese hamster ovary(CHO) cells	Increases	61,65,66
Human malignant melanoma (M) cells	Increases	61
Novikoff hepatoma cells	Increases	7

A.2.4.4.2 Cellular metabolism

Mammalian cell lines have been shown capable of metabolising DTIC particularly in the absence of light (61,65). Incubation of ^{14}C-methyl labelled DTIC with Chinese hamster ovary (CHO) cells resulted in association of radioactivity with DNA only in the dark, where metabolism would yield the methylating agent, MTIC, and $^{14}CO_2$ was detected (61). In separate experiments, AIC was characterized in CHO cells after incubation with DTIC in the dark (65). Human tumour slices and sarcoma 180 (S180) were also able to metabolise ^{14}C-methyl labelled DTIC with production of $^{14}CO_2$ (67). *In vitro* alkylation of DNA and RNA by DTIC occurred upon incubation with S180 tumour microsomal preparations, and formaldehyde and AIC were produced (67). In contrast, Hill found no detectable demethylating activity in S180 tumour fractions (43). Such direct evidence for DTIC N-demethylation indicates a rôle for this metabolic pathway (as well as the photodecomposition pathway) in the *in vitro* cytotoxicity of DTIC.

A.2.4.4.3 The influence of hypoxic conditions

Cytotoxicity of DTIC against CHO and HeLa cells protected from the light was compared under aerobic and hypoxic conditions (68). Hypoxic CHO cells were 5-6 times more sensitive than aerobic cultures to the cytoxicity of high concentration DTIC at 37^{o}C. The sensitivity was reduced to the low value of that in aerobic cells on lowering the incubation temperature, suggesting the need for DTIC activation. *In vivo*, DTIC was slightly more toxic towards KHT

cells in irradiated mice (hypoxic survivors) than to those in untreated mice. There is a possibility of selective activation under hypoxic conditions, perhaps by a reductive process as has been described for phenyltriazenes (69). Such a mode of action is obviously important both in the treatment of irradiated tumours, and also in solid tumours where many of the cells do not have an adequate oxygen supply.

A.3 Structure - Activity Relationships for Triazenes

A.3.1 Requirement for a methyl group

Hano *et al*, looking at the antitumour activity of dialkyltriazenylimidazolecarboxamides against Ehrlich solid carcinoma in mice, noted that the dimethyl derivative had the highest therapeutic effect; increasing the alkyl group chain length decreased activity while toxicity increased (10). A further triazene series with differing alkyl groups showed greatest antitumour activity where there was at least one methyl group. Hansch *et al* also analyzed structural requirements working with L1210 leukemia in mice and confirmed the necessity for a methyl group (70). For the second alkyl group, longer chains promote activity; 3-methyl-3-pentyl derivatives have been found particularly active against both L1210 and TLX5 tumours *in vivo* (71,72).

Audette and co-workers showed that a series of aryldimethyltriazenes was active, whereas in general aryldiethyltriazenes were inactive *in vivo* against TLX5 lymphoma (53). The interpretation of these and other results was that only compounds comprising one methyl group and a preferentially metabolised group are active, at least against TLX5 cells (45,72) (figure 5). For each compound, activity is seen only where a monomethyltriazene may be produced on metabolism. For example, the methyl-*tert*-butyl derivative is inactive since the *tert*-butyl group has no α-CH bond and cannot be dealkylated to yield the monomethyltriazene (45,73). There are some notable exceptions to this rule however, with diethyltriazenes exhibiting antitumour activity *in vivo* against L1210 leukemia (74).

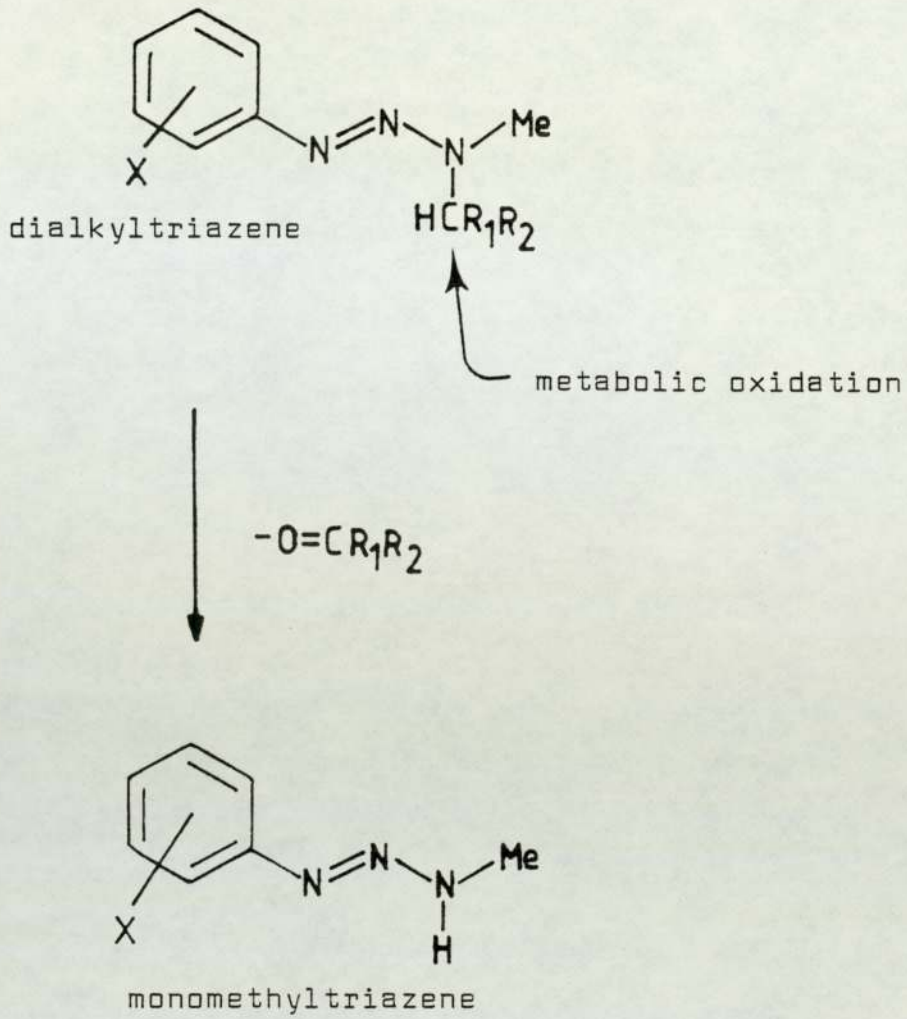


Figure 5 Requirement for the production of a monomethyltriazene during metabolism for antitumour activity.

A.3.2 Identity of the aryl- or heteroaryl- group

Hansch and co-workers evaluated a series of phenyl-, pyrazolyl- and imidazole- triazenes against L1210 leukemia in mice (73). An ideal lipophilicity was established for antitumour activity ($\sim$ log P 1·1), with no additional potency associated with a heterocyclic ring, or ortho-substitution of an aryldimethyltriazene. Activity was dependent on the electronic characteristics of the aryl substituent. The relationship predicted the futility of any endeavour to

synthesize more potent aryltriazenes, since electron-releasing substituents enhance activity, but result in unstable compounds. In addition, Hansch failed to discover leads pointing to less toxic rather than more potent triazenes, and thus obtain derivatives with a better therapeutic index (74).

Dunn et al working with S180 ascites tumour in the mouse, found the lipophilic nature of the ring substituent in a series of aryldimethyltriazenes to be of no significance, but its electronic nature explained 85% of the variance in antitumour activity (75). There was a strong correlation between cytotoxicity and the rate of protolysis of the aryldimethyltriazenes to the diazonium cation (figure 6). The study indicated that, for this activation mechanism, there was difficulty in separating the toxic and antitumour properties of triazenes (75).

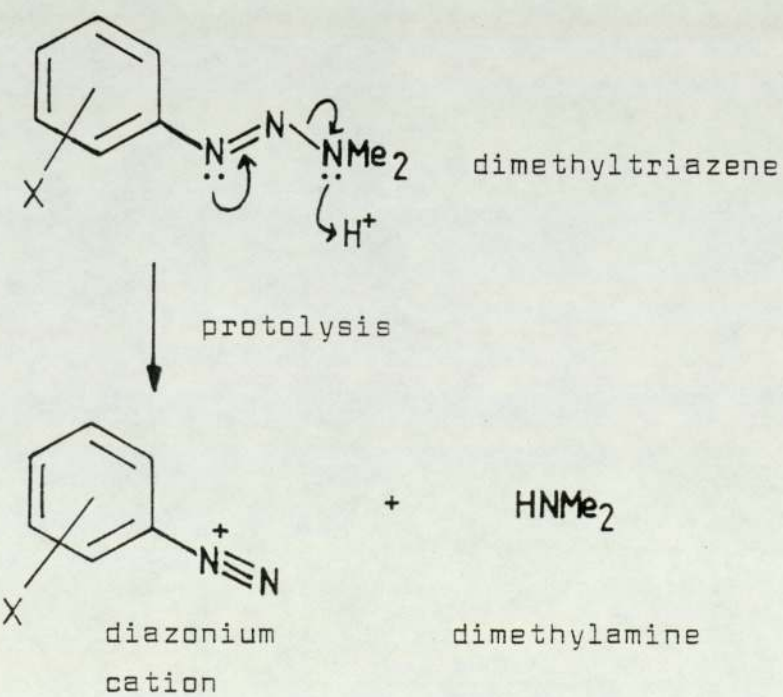


Figure 6 Protolysis of a dimethyltriazene

In contrast, Connors *et al* observed little variation in activity against TLX5 lymphoma *in vivo* with a change in phenyl ring substitution (45).

A.3.3 Evaluation of structure-activity relationships

Interpretation of screening work with L1210 leukemia and TLX5 lymphoma resulted in agreement upon the requirement for a methyl group for activity, and yet there are other triazene derivatives, such as diethyltriazenes that are active antitumour compounds. The effect of phenyl ring substitution was variable, depending on the tumour line used. Results could be influenced by the great sensitivity of both L1210 and TLX5 tumours to cytotoxic species, such as the monomethyltriazene, as distinct from any selective antitumour species since these tumours will preferentially respond to triazenes which may be activated to yield alkylating species. The S180 cell line does not show this preferential sensitivity, and antitumour activity seems more linked to diazonium ion production from triazenes. This mechanism cannot be dismissed even though diazonium compounds appear to be inactive *in vivo* (53), since the dimethyltriazene may be transferred to the cell before undergoing protolysis, whereas an administered diazonium compound may have little chance of entering the intact tumour cell (75).

It would appear that depending on the test system used, different structural requirements for active triazenes are obtained.

A.4 Triazenes as Alkylating Agents

A.4.1 Isolation of methylated products

Table 2 summarizes both the *in vivo* and *in vitro* results obtained using ^{14}C-methyl labelled triazenes. 7-Methylguanine has frequently been isolated as a methylated product - there is no evidence for its occurrence normally in mammalian DNA (76). Where guanine and adenine were radiolabelled (table 2), it was with a much lower specific activity suggesting their formation was via incorporation of $^{14}CH_2O$ into the one carbon pool with subsequent *de novo* purine biosynthesis.

In an assay for *in vivo* alkylation based on the degree of S-methylation of cysteine in haemoglobin, no methylation was observed using DTIC, although a low level could be masked by naturally occurring S-methylcysteine in the rat (81). *In vitro* experiments with a phenylmonomethyltriazene (Ig) confirmed the low methylating activity of triazenes in this system - the levels of methylation were only 5% of those elicited by methyl methanesulfonate, a directly acting alkylating agent.

A.4.2 Mechanism of methylation

The N-demethylated product of DTIC, MTIC, is unstable in solution and methylates nucleophiles by an SN_2 reaction yielding AIC (figure 7). Incubation of deuterated MTIC with DNA further demonstrated that the methyl group is transferred intact and that there is no involvement of diazomethane (82).

Table 2 *In vivo* and *in vitro* methylation by triazenes

Experimental system employed	Triazene used	Labelled product	Isolation site	Reference
S180	DTIC	7-methyl-guanine	nucleic acid hydrolysate	67
In vivo rats and man	DTIC	7-methyl-guanine (adenine)* (guanine)*	urine. RNA, DNA of rat tissues	77
rat liver microsomes	DTIC	7-methyl-guanine	RNA, guanosine	77
in vivo rats	(Ia)	7-methyl-guanine	RNA, DNA of liver, kidney small amounts in brain, spleen, small intestine	78 79
mouse fibroblast (L) cells	MTIC	7-methyl-guanine	DNA	80

* lower specific activity

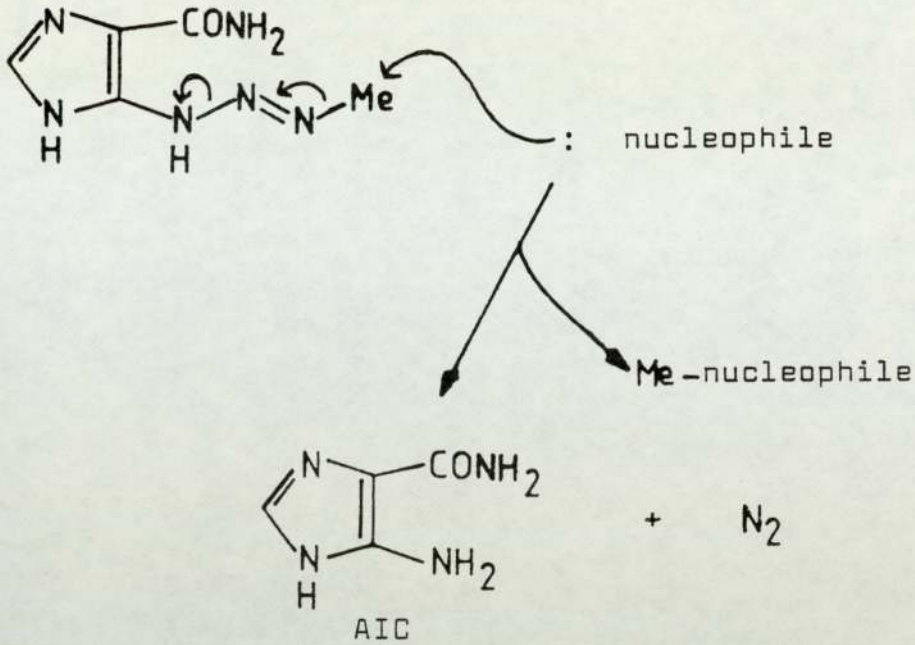


Figure 7 Mechanism of methylation by MTIC

A.4.3 Comparative actions - alkylating agents and triazenes

The sensitivity of resting and dividing bacterial cells (E.coli) to anticancer drugs is a good model for activity against tumour cells (83). DTIC was found to be 56 times more active against dividing than resting cells, in contrast to alkylating agents where the increased sensitivity was only by a factor of 6 and BCNU by 13. These results suggest that DTIC does not act as a typical alkylating agent.

TLX5 lymphoma is not sensitive to classical alkylating agents of the β-chloroethyl type, and yet is sensitive to triazenes. The PC6 tumour is also sensitive to the antitumour activity of triazenes, although it is resistant to monofunctional alkylating agents, examples of which are the monomethyltriazenes, the proposed active alkylating species produced from dimethyltriazenes (53). BCNU is a nitrosourea which possesses alkylating activity yet it differs from classical alkylating agents in a mechanistic sense (84). A TLX5

cell line made resistant to a dimethyltriazene (VIIa) *in vivo* was cross resistant to other dimethyltriazenes and BCNU, but not to the antimetabolites methotrexate and cytosine arabinoside (53).

An L1210 line made resistant to BIC, was also resistant to DTIC and nitrosoureas (85). In contrast, L1210 cells made resistant to DTIC retained sensitivity to BIC, a variety of alkylating agents, and also to nitrosoureas (71,86). The BCNU results are contradictory, but it would appear that BIC is more closely related to alkylating agents, and acts by a different mechanism to DTIC.

Alkylating agents such as cyclophosphamide interfere with the G_2 phase of the cell cycle (87). Results with DTIC have been variable, both for and against an alkylating mechanism (66,86).

Many workers have studied the effect of triazenes on the incorporation of radioactive precursors into DNA, RNA and protein despite evidence that this may not accurately reflect macromolecular synthesis (88). Alkylating agents with a primary action on DNA selectively inhibit thymidine incorporation into DNA (53). Antimetabolites, such as 6-mercaptopurine which interfere with purine biosynthesis, have an inhibitory effect on DNA and RNA, but not protein. Table 3 summarizes the results obtained with triazenes. Some of these results imply that triazenes act as alkylating agents, while others do not. Experiments with DTIC carried out in the light probably reflect the action of diazo-IC, rather than that of the parent compound.

Table 3 Effect of triazenes on radioactive precursor incorporation into macromolecules

Triazene	Cell system	Site of incorporation inhibition	Reference
(VIIa)	TLX5	DNA RNA protein	53
DTIC (hv)	L1210	protein= RNA > DNA	86
DTIC (hv)	B.subtilis	DNA	59
DTIC (hv)	E.coli	DNA	60
DTIC- in vivo	Regenerating liver, spleen thymus, small intestine	DNA (protein)*	89
MTIC	mouse fibroblast (L) cells	DNA> RNA	80

* lesser effect

A.4.4 Further evidence against methylation as the mechanism of triazene antitumour action

Methylation of DNA has been shown to induce single strand breaks, thus reducing the ability of DNA to act as a template for DNA and RNA synthesis (90), an effect that could result in cell kill. One problem is the lack of specificity of the alkylating process. Activated DTIC or MTIC cause methylation in both normal (77) and tumour tissue (67) and even *in vitro* (91). The only way for a selective cytotoxicity to occur would be if in normal tissue there was preferential repair of lesions.

Human malignant melanoma cells (M cells) both sensitive and resistant to DTIC showed the same radiolabelling of nucleic acids upon incubation with DTIC (61). Methylation or, in general, alkylation cannot explain the apparent need for one methyl group for activity (53), since both monomethyl- and monoethyl- triazenes alkylate *in vitro* (92). Both dimethyl- and diethyl- triazenes are dealkylated (41,53) and are equitoxic to the animal (45,53), yet only the dimethyltriazenes have *in vivo* antitumour activity.

It is possible that the cytotoxic species formaldehyde liberated from active triazenes is important for *in vivo* activity, whereas higher aldehydes yielded from inactive triazenes are not cytotoxic. However, experiments on the antitumour activity of combinations of formaldehyde and inactive but dealkylated triazenes have produced negative results (53). Also hydroxymethyltriazenes (Ie) which can act as formaldehyde pro-drugs behave biologically like monomethyltriazenes both *in vitro* and *in vivo* (51).

Gescher *et al* demonstrated the selective cytotoxicity of a dimethyl- but not a diethyl-triazene to sensitive TLX5 cells as compared with a TLX5 line made resistant to the activity of dimethyltriazenes *in vivo* (47). The corresponding monomethyltriazene (Iy) was equitoxic to both cell lines *in vitro* suggesting that it is not responsible for selective anti-tumour activity. In an *in vivo* test, the resistant line was resistant to the monomethyltriazene (Iy) and it was proposed that the triazene may be further activated by the host (48).

A.4.5 Carcinogenicity and mutagenicity of triazenes

A.4.5.1 Distant and proximate tumourigenicity

Alkylation by triazenes is more clearly linked to their carcinogenicity. DTIC and dimethyl- and diethyl-phenyltriazenes are distant carcinogens in rats, only very infrequently causing a sarcoma at the injection site (93). Phenylmethyltriazene (Ib) is a powerful local carcinogen causing tumours in the forestomach and oesophagus on oral administration, and local sarcoma after injection, but also, like the dimethyltriazene (Ia), some distant neurogenic tumours (93). DTIC is teratogenic in both the rat and rabbit (94,95).

That the dialkyltriazenes do not produce local tumours suggests that they are not directly acting carcinogens but need activation. As there are no stomach tumours produced on oral administration (93,96), this rules out the diazonium compound, which would easily form in the stomach acid, as the proximate carcinogen. Monoalkyltriazenes are likely candidates since they are direct and locally acting carcinogens. Many classes of compounds are carcinogenic because of their alkylating ability.

A.4.5.2 Triazene transport forms

Dimethyltriazenes are metabolised particularly in the liver, and yet cause tumours in the central nervous system for example. There is some evidence that monomethyltriazenes can cause tumours at distant sites (93,97) and may be transport forms. Another possibility is the hydroxymethyltriazene (Ie) first suggested by Preussmann *et al* (41). Recent metabolic studies with 1-phenyl-3,3-dimethyltriazene (Ia) have identified 1-phenyl-3-acetyl-3-methyltriazene (Id) as an *in vitro* metabolite (69). This could be transported away from the liver and deacetylation at the site of action would yield the carcinogenic monomethyltriazene.

The nature of the transportable form of a triazene has implications for antitumour activity as well as carcinogenicity, since the tumour target site will, in the majority of cases be distant from the liver where activation of the compound occurs.

A.4.5.3 Mutagenic activity

There have been numerous reports of the mutagenicity of triazenes in different test systems, both with and without activation (98-103). 1-Phenyl-3,3-dimethyltriazene (Ia) was positive in a short term screening test for carcinogenicity - the ability to cause an increase in chromosomal aberrations in rat lymphocytes cultured *in vitro* after *in vivo* drug treatment (104).

A quantitative structure-activity study by Hansch and co-workers demonstrated that the mutagenicity of triazenes (measured using the Ames test) could be minimized with little loss of antitumour activy (105). Further studies based on molecular shape analysis confirmed this observation (106).

A.5 Other Triazene Properties

A.5.1 Triazenes as radiosensitisers

As reactive species can be so easily generated from DTIC by photolysis, there has been recent interest in any similar activation by radiation, and particularly detection of any radiosensitising effect. DTIC combined with ^{60}Co -γ- irradiation has been shown to be more effective than radiation alone in inhibiting melanoma growth in nude mice (107), but there was no evidence for this type of effect in CHO or HeLa cells *in vitro* at non-toxic concentrations of DTIC (68).

A.5.2 Enzyme inhibition by triazenes

A.5.2.1 Xanthine Oxidase

Purine analogues are known inhibitors of xanthine oxidase, the enzyme which catalyses conversion of hypoxanthine via xanthine to uric acid. 2AH and AIC, and DTIC in the dark were not active (108-110), but diazo-IC did cause inhibition of this enzyme (109). Measurements of serum and urine uric acid in patients treated with DTIC did not reveal decreased uric acid production which would accompany the oxidase inhibition (22). Both diazo-IC and 2AH also inhibitied uricase *in vitro* (110).

A.5.2.2 Phosphodiesterase

There is some evidence that high levels of cyclic 3′,5′-adenosine monophosphate (cAMP) will cause a reduction in cellular proliferation. DTIC was found to elevate cAMP in hepatocytes and hepatoma cells by inhibition of phosphodiesterase, the enzyme which breaks down cAMP (111,112) and such an effect *in vivo* would be additive with other

mechanisms of tumour cell kill. An antitumour mechanism involving cAMP phosphodiesterase is also a possibility for various alkylating agents (113).

A.5.3 Immunopharmacology of triazenes

The immunopharmacology of DTIC has been reviewed by Fioretti (114). The treatment of L1210 leukemia *in vivo* with DTIC in order to produce resistant cells causes an increase in tumour cell immunogenicity, so that an increase in survival time of the tumour bearing animals is seen (71), and eventually in some cases tumour rejection may occur (115). In contrast, mice bearing the same triazene altered cells, and subsequently treated with DTIC, died earlier than control untreated mice, possibly due to the immunosuppressive action of the drug (115). Since, *in vitro*, mouse liver microsomes must be present in order for DTIC to produce the immunogenic leukemia, it follows that a DTIC metabolite is responsible for this effect (116).

Similar immunogenic changes were produced using dimethyl- and monomethyl- aryltriazenes (117), and it was shown that there was no correlation between antitumour activity and production of immunogenic cells. Work with phenyltriazenes suggested the importance of monomethyltriazenes in the generation of increased immunogenicity (44).

The immununogenic changes are heritable over several transplant generations and not the result of DTIC or DTIC metabolites bound to the cell membrane (118). A study involving injection of leukemic cells into X-irradiated golden hamsters, revealed a loss of oncogenic potential in

triazene treated L1210 lines (119). The mechanism for the decrease in oncogenicity or immunogenic changes is unknown, neither is the relevance of the effect to DTIC therapy.

A.5.4 Antimetastatic triazenes

The first reported selectively antimetastatic triazene was 4-carbethoxy-5-(3,3-dimethyl-triazen-1-yl)-2-methylimidazole (VIIb) which inhibited formation of pulmonary metastases in mice bearing Lewis lung tumours (120). Giraldi and co-workers compared activities of (VIIb), DTIC and a phenyldimethyltriazene (Ih) (121). All three compounds reduced the number of metastases from Lewis Lung tumour, with marginal activity against the primary tumour shown only by DTIC and (VIIb). The result suggested an activation mechanism for the antimetastatic action differing from the mechanism responsible for primary tumour inhibition. Diazonium compounds were not active as antimetastatic agents, whereas another possible hydrolysis product of (Ih), (Ii) and its potassium salt were effective (122).

In a study of para-substituted phenyldimethyltriazenes (123), activity against the primary tumour was related to drug stability - more stable triazenes being more susceptible to N-demethylation (56). The antimetastatic activities of all the triazenes were however equivalent, again suggesting an activation mechanism other than N-demethylation is required to produce the antimetastatic species. In confirmation of this, it was observed that the products of N-demethylation,

monomethyltriazenes,are less active than the parent dimethyltriazenes as antimetastatic agents (123). Furthermore, the potassium salt of (Ii) is a selective antimetastatic agent in mice bearing Lewis lung tumour, yet it is not N-demethylated (123).

Pretreatment of mice with an antimetastatic triazene prior to transplantation of the primary Lewis lung tumour resulted in an increase in the total number of metastases (124). It is therefore likely that the site of action is a direct effect on tumour cells, rather than involvement of the host by making conditions less favourable for metastasis formation. The ineffectiveness of antimetastatic triazenes against intra-venously injected artificial metastases (124), indicated that lodgement of cells in the lung is not the triazene site of action. The mechanism of action may therefore be inhibition of an early stage in metastases formation such as entry of cells into the blood stream.

Sava and co-workers studied antimetastatic triazenes as adjuvants to surgery (125). Surgery alone was not curative against Lewis lung carcinoma implanted into the hind leg of mice, but drug plus surgery produced some long term survivors. Triazenes therefore proved their effectiveness as prophylactic adjuvants to surgery and may be clinically useful in combination with other agents active against the primary tumour.

A.6 Conclusion and aims of the present work

Even after a considerable amount of work by chemists and biochemists over many years, there is still great controversy over the mode of action of antitumour triazenes. The activity of such a vast range of compounds with only the triazene linkage in common makes it unlikely that they act by inhibition of purine biosynthesis, which was the reason why many of them were first synthesized.

There is much evidence to suggest that dimethyltriazenes must be activated in some way to be selective antitumour agents, but the identity of the active species is still not clear. It could be that it varies depending upon conditions and the tumour challenge presented, or that on each occasion activity is seen only because of a combination of effects of different active species. For example, it would seem that activation by hydrolysis is required for activity against the S180 tumour and by N-demethylation for TLX5 cell kill. Also a different activation mechanism has been proposed to produce activity against the primary tumour as distinct from an antimetastatic effect.

One of the most widely-supported mechanisms for the antitumour activity of triazenes is the methylation hypothesis. Metabolites of dimethyltriazenes do methylate macromolecules, and are active agents, but there are many results contradictory to this theory. In fact, it is more likely that this mechanism explains the cancer causing rather than the cancer curing properties of the triazene series.

The aim of the work described in this thesis was to study more closely the triazene molecule from both a chemical and biological standpoint. The work is grouped into four sections:

i) Stability studies

a) To look at the photodecomposition products of DTIC under different conditions in comparison with those of hydrolysis of diazo-IC, suggested as a possible activated triazene species, and to check likely chemical interactions during drug administration.

b) To determine the stability of dimethyltriazene metabolites, and therefore to assess the likelihood of their being transported from the liver to the site of antitumour activity.

ii) Metabolism studies

a) To look at metabolism of phenyldialkyltriazenes particularly under conditions where selective antitumour activity has been demonstrated, in order to assess the extent of N-dealkylation and production of various metabolites.

b) To compare the rates of hydrolysis and metabolism of the alkylating species monomethyl- and monoethyl- triazenes with the aim of determining why only monomethyltriazenes are active antitumour agents *in vivo*.

c) To detect any metabolism of a monomethyltriazene by TLX5 cells, a possible mechanism of drug resistance.

iii) Toxicity studies

a) To compare toxicity of a dimethyl- and a monomethyl-triazene in an hepatocyte system where both compounds may be metabolised leading either to activation or deactivation.

b) To determine any differential toxicity of a monomethyltriazene to triazene sensitive and resistant TLX5 cell lines in order to assess this agent as the selectively active species.

iv) Glutathione studies

Glutathione is ever present in biological systems. Its status in the cell is known to influence drug metabolism and action, and, conversely, a wide range of compounds themselves affect glutathione. It was therefore of interest to ascertain any interaction between triazenes and this important tripeptide under varying conditions:

a) To determine whether glutathione levels are responsible for the development of triazene resistance in TLX5 tumour cells.

b) To study the effect of triazenes on glutathione levels in mouse hepatocytes.

c) To detect any triazene-induced inhibition of hepatocyte glutathione-reductase, the enzyme which keeps glutathione predominantly in the reduced state - the form in which it is used in detoxification reactions.

d) To look at the influence of triazene derivatives on the oxidation state of glutathione in mouse liver homogenates.

PART B

MATERIALS

PART B MATERIALS

B.1 Triazenes and related compounds

B.1.1 Purchased

5-Aminoimidazole-4-carboxamide:- Aldrich Chemical Company, Gillingham.

4-Aminoacetophenone:- B.D.H. Chemicals Limited, Atherstone.

4-Aminobenzonitrile:- Ralph N. Emanuel Limited, Wembley.

Methyl-4-aminobenzoate:- Koch-Light Laboratories Limited, Colnbrook.

B.1.2 Gifts

DTIC-Dome was obtained from Dr. J. G. Goodall, Miles Laboratories Limited.

5-(3,3-Dimethyltriazen-1-yl)imidazole-4-carboxamide was a gift from Dr. Harry B. Wood, National Cancer Institute, Bethesda, Maryland, U.S.A.

4-Carbamoylimidazolium-5-olate was provided by Dr. Kimio Mizuno of Toyo Jozo Company Limited, Tokyo, Japan.

B.1.3 Synthesized

Compounds were synthesized in our laboratories by:

i) Prof. M. F. G. Stevens

ii) Dr. R. J. Simmonds

iii) Dr. M. D. Threadgill

iv) Dr. K. Vaughan

v) Mrs. G. U. Baig

Diazo-IC and 2AH were synthesized by the method of Shealy et al (9).

Aryltriazene derivatives, some of which were novel compounds, were synthesized by the general method of coupling an aryl-

diazonium salt with the appropriate primary or secondary aliphatic amine (53, 126 - 129).

B.2 Other cytotoxic drugs

The cytotoxic drugs used in section D.1.2.2 were clinically used formulations and a gift from the General Hospital, Birmingham.

BCNU used in section D.4.3 was a gift from the National Cancer Institute, Bethesda, Maryland, U.S.A.

B.3 Miscellaneous chemicals

The following compounds were purchased from the sources indicated.

B.3.1 Aldrich Chemical Company, Gillingham

Glutathione, reduced 96% G470-5

2-Vinylpyridine

B.3.2 B.D.H. Chemicals Limited, Atherstone

Acetylacetone

Dimethyldichlorosilane solution 2% in 1,1,1-trichloroethane

Disodium EDTA

Folin and Ciocalteu's phenol reagent

Metaphosphoric acid (60% HPO_3 + 40% $NaPO_3$)

NEDA

Paraformaldehyde

Trisodium orthophosphate

B.3.3 Fisons Scientific Apparatus Limited, Loughborough

Ammonium acetate

B.3.4 May and Baker Limited, Dagenham

Sagatal (Pentobarbitone sodium BP vet) 60 mg ml^{-1}.

B.3.5 Sigma Chemical Company, Poole

Bovine Serum Albumin	A-8022
Diethyldithiocarbamic acid	D-3506
EGTA	E-4378
Glucose-6-phosphate	G-7870
Glutathione, oxidized, disodium salt	G-4626
Heparin	H-7005
HEPES	H-3375
NADP	N-0505
NADPH	N-1630
Trizma base	T-3253

B.3.6 Vestric Limited (Knights Birmingham Branch)

Phenobarbitone sodium BP

B.4 Enzymes

The following enzymes were obtained from Sigma Chemical Company, Poole.

Collagenase, type IV	C-5138
Glucose-6-phosphate dehydrogenase type XI	G-8878
Glutathione reductase type III	G-4751

B.5 Chromatographic materials

B.5.1 Columns and plates

Standard HPLC columns were purchased from Anachem Limited, Luton.

Radial Compression columns were purchased from Waters Limited, Northwich.

Thin layer chromatography plates (Kieselgel 60F 254, Merck Art 5735) and silica gel G(Typ 60) (Merck) and alumina GF_{254} (Typ 60/E) (Merck) for tlc plate preparation were obtained from B.D.H. Chemicals Limited, Atherstone.

B.5.2 Solvents

HPLC grade chloroform, dichloromethane and acetonitrile were obtained from Fisons Scientific Apparatus Limited, Loughborough. Dimethylamine was purchased from B.D.H. Chemicals Limited, Atherstone.

Analar grade toluene and acetone were obtained from B.D.H. Chemicals Limited, Atherstone.

B.6 Animals and their diets

Bk/Wistar rats and CBA/Lac and CBA/Ca mice were obtained from Bantin and Kingman Limited, Hull and were maintained in the University animal house for one week prior to use. They were fed on Heygates modified 41B breeding diet and allowed access to water ad libitum.

B.7 Buffers and incubation media

B.7.1 For photodecomposition and stability studies

Buffers were prepared from stock solutions A and B as described in Documenta Geigy (130).

i) Clark and Lub's potassium chloride-hydrochloric acid pH 1+2

A - potassium chloride 0.2N

B - hydrochloric acid 0.2N

ii) Sørensen's glycine I

pH 2.5+3.

A - hydrochloric acid 0.1N

B - glycine 0.1M in sodium chloride 0.1N

iii) Sørensen's phosphate

pH 5.2 + 7.4.

A - potassium dihydrogen phosphate 9.073 g L^{-1}

B - disodium hydrogen phosphate dihydrate 11.87 g L^{-1}

iv) Sørensen's glycine II

pH 8.5 + 10.15 + 12.

A - glycine 0.1M in sodium chloride 0.1N

B - sodium hydroxide 0.1N

B.7.2 Earl's buffer

Sodium chloride	6.80 g
Sodium bicarbonate	2.20 g
Glucose	1.00 g
Potassium chloride	0.40 g
Sodium dihydrogen phosphate dihydrate	0.14 g
Distilled water	to 1 litre.

Buffer adjusted to pH 7.4 by addition of hydrochloric acid.

B.7.3 Tris buffer 0.01M

Trizma base	1.21 g
Distilled water	to 1 litre.

Buffer adjusted to pH 7.4 by addition of hydrochloric acid.

B.7.4 Krebs-Henseleit buffer

(for hepatocyte and TLX5 cell incubation).

Sodium chloride	6.87 g
Sodium bicarbonate	2.10 g
Glucose	2.00 g
Potassium chloride	0.40 g
Magnesium sulphate heptahydrate	0.14 g
Calcium chloride	0.28 g
Sodium dihydrogen phosphate dihydrate	0.14 g
Distilled water	to 1 litre

Buffer adjusted to pH 7.4 by addition of hydrochloric acid.

B.7.5 Sodium phosphate 1.25 mM, sodium EDTA 6.3 mM

(for glutathione assay).

Disodium hydrogen phosphate dihydrate	11.12 g
Disodium EDTA	1.172 g
Distilled water	to 500 ml

Buffer adjusted to pH 7.5 by addition of hydrochloric acid.

B.7.6 Calcium free HEPES buffer

(for preparation of mouse hepatocytes).

Sodium chloride	8.3 g
Potassium chloride	0.5 g
HEPES	2.4 g
Sodium hydroxide 1N	5.5 ml
Distilled water	to 1 litre

Buffer adjusted to pH 7.4 by addition of hydrochloric acid.

B.7.7 Potassium phosphate buffer containing 0.2 M potassium chloride and 1 mM EDTA

(for glutathione reductase assay).

Potassium dihydrogen phosphate	1.395 g
Dipotassium hydrogen phosphate	2.305 g
Potassium chloride	7.455 g
EDTA	0.146 g
Distilled water	to 500 ml

Buffer adjusted to pH 7.4 by addition of hydrochloric acid

8.7.8 Horse serum

This was purchased from Gibco Limited, Glasgow.

8.8 Miscellaneous reagent solutions

8.8.1 Nash reagent

Ammonium acetate	112.5 g
Acetylacetone	1.5 ml
Acetic acid	2.25 ml
Distilled water	to 250 ml

8.8.2 tlc spray reagents

i) NEDA

NEDA	700 mg
Concentrated hydrochloric acid	65 ml
95% ethanol	285 ml

ii) Dimethylaminobenzaldehyde

4-Dimethylaminobenzaldehyde	700 mg
Concentrated hydrochloric acid	65 ml
95% ethanol	285 ml

PART C

METHODS

PART C METHODS

C.1 Stability studies

C.1.1 Spectroscopic-scale photolysis

Photolysis was carried out in 1 cm quartz cuvettes exposed to sunlight, with controls kept in the dark. Soluble compounds were dissolved directly in the photolysis buffer; DTIC was dissolved first in DMSO and phenyltriazenes in acetonitrile and diluted with buffer to a concentration of approximately 0.01 mg ml^{-1} prior to photolysis. Spectral scans were taken between 450 and 200 nm (Unicam SP 8000 Spectrometer) at different time intervals depending on the stability of the test compound, and photolysates were compared with reference spectra at the appropriate pH.

C.1.2 Half-life determinations

Spectroscopic scale decomposition of samples kept in the dark in the spectrometer maintained at 37°C was monitored by scans taken at time intervals determined by a Unicam SP 8005 programme controller operating in the repeat scan mode. The decrease in absorbance with time was measured at the λ max of the compound under study and the half-life calculated from the slope of a logarithmic plot of the extent of decomposition on the ordinate against the time of hydrolysis on the abscissa.

HPLC determinations of half-life are described in section C.2.5.4.

C.1.3 Preparative-scale photolysis

DTIC-Dome was dissolved at a concentration of 10 mg ml^{-1} water in a 100 ml volumetric flask, and exposed to direct

sunlight. pH measurements and spectral examination of diluted samples of the photolysate were made during the investigation.

C.2 Metabolism studies

C.2.1 Liver preparation

Animals used were female Bk/Wistar rats (150-200g) and both male and female CBA/Lac or CBA/Ca mice (20-25g).

Livers were excised, weighed and washed with 0.25M sucrose. A 25% $^{w}/_{v}$ homogenate was made in ice-cold sucrose using 8 strokes of a Camlab 563C homogeniser (speed 5) fitted with a teflon pestle. The resulting suspension was centrifuged at 9000g for 20 minutes in a MSE/Fisons Hi-spin 21 centrifuge at 4^{o}C. The 9000g supernatant was used directly, or further centrifuged by the method of Schenkman and Cinti (131) to prepare microsomes. This method utilizes calcium chloride to increase the microsomal sedimentation rate, and it has been shown that the resulting microsomes have mixed function oxidase enzyme activity very similar to that of conventionally prepared microsomes (132). The microsomal supernatant was used directly in some experiments and the pellet was resuspended in Earl's buffer to the same volume as the 9000g homogenate from which it was prepared.

C.2.2 Phenobarbitone induction

Phenobarbitone was administered to rats in the drinking water at a concentration of 500 mg L^{-1} for three days prior to sacrifice (45).

C.2.3 Protein content of microsomes

This was estimated by the method of Lowry et al (133).

C.2.3.1 Solutions

a) Alkaline copper sulphate solution - 50 ml A + 1 ml B.

Solution A - 2% sodium carbonate decahydrate in sodium hydroxide 0.1N.

Solution B - equal parts of solutions B_1 + B_2.

Solution B_1- 1% copper sulphate pentahydrate.

Solution B_2- 2% sodium potassium tartrate.

b) Folin's reagent - Folin and Ciocalteu's phenol reagent diluted with an equal volume of distilled water.

C.2.3.2 Assay method

Microsomal sample (1ml) diluted to a liver concentration of 8mg ml^{-1} was added to 5ml of alkaline copper sulphate solution. After 10 minutes at room temperature, 0.5ml of Folin's reagent was added and the solutions mixed immediately. After a further 30 minutes at room temperature, absorption readings were taken at 750nm using a Cecil CE 5095 spectrophotometer.

A protein calibration curve was constructed using BSA at concentrations of 0-300 μg ml^{-1}.

C.2.4 TLX5 ascites cell preparation

The TLX5 lymphoma was passaged at 7 day intervals by i.p. injection of approximately 2×10^5 cells into 20g male CBA/Lac or CBA/Ca mice. The TLX5R lymphoma passaged in the same way had been made resistant to a dimethyltriazene (VIIa) *in vivo* (53), and has been shown to be resistant to the optimum antitumour dose of all other dimethyltriazenes. Cells were counted in a model ZBI Coulter counter.

Ascites cells of both types were harvested from a routine passage and suspended in Earl's buffer pH 7.4 at 2×10^6 cells per ml, ready for incubations.

C.2.5 Incubations

Unless otherwise stated, metabolic incubations were carried out in 25ml pyrex beakers exposed to atmospheric air in a shaking water bath (80 strokes/min) at 37°C. Cofactors, including components of an NADPH generating system were dissolved and incubates made up to volume using Earl's buffer pH 7.4. Incubation conditions for the different experiments are given in table 4.

C.2.5.1 Preliminary metabolism studies on AcDMT

Metabolism was carried out in 50ml beakers, and incubates were made up in phosphate buffer pH 7.4. Metabolism was started by addition of substrate in 100 µl acetone, and stopped by addition of 2 ml each of saturated barium hydroxide and 20% zinc sulphate solution.

C.2.5.2 Metabolism of AcDMT under the conditions of the bioassay and comparison with aminopyrine

The disappearance of AcDMT was monitored with time, with incubations conducted in closed 20 ml Universal containers as well as in 25 ml open beakers. Metabolism was initiated by addition of triazene in 50 µl DMSO, and samples were extracted for assay at time intervals. The disappearance of aminopyrine (50 µM) added dissolved in buffer was followed by a similar method, except the volume of liver preparation was doubled in order to obtain a detectable level of metabolism.

For the determination of aminopyrine (4mM) metabolism by the Nash formaldehyde assay, incubates were deproteinised after 30 minutes by addition of 0.25 ml 20% TCA and centrifugation for 5 minutes.

Table 4 Metabolic Incubation Conditions

Substrate	Animals *	Liver homogenate(ml) 9000g	microsomes	cofactors(mg) G6P	NADP	$MgCl_2$	G6PdH(u)	Final volume	Incubation time (min)	Analysis
AcDMT 5 mg in 13 ml	R	10	-	100	10	50	15	13	30	tlc
AcDMT 50 μM	R Ma,Mb	0.4	0.4	20	2	6	1	2.0	up to 120	normal phase HPLC
Aminopyrine 50 μM	R Ma,Mb,Md	0.8	0.8	20	2	6	1	2.5	30	normal phase HPLC
Aminopyrine 4 mM	R Ma,Mb,Md	0.8	0.8	20	2	6	1	2.5	30	Nash assay
Dialkyltriazenes 500 $\mu g\ ml^{-1}$	Mc	0.4	-	3.1	0.84	2.75	1.5	2.0	up to 120	reverse phase HPLC
Monoalkyltriazenes and arylamines 4 $\mu g\ ml^{-1}$	Mc	0.8	-	3.1	0.84	2.75	1.5	2.5	up to 30	reverse phase HPLC
AcMMT, AcMET CyMMT, CyMET 50, 100 $\mu g\ ml^{-1}$	Mc	0.8	-	3.1	0.84	2.75	1.5	2.5	up to 30	reverse phase HPLC

* R Female Bk/Wistar rat
Ma Male CBA/Lac mouse
Mb Female CBA/Lac mouse
Mc Male CBA/Ca mouse
Md Female CBA/Ca mouse

C.2.5.3 Quantification of products formed during metabolism of dialkyltriazenes

Metabolism was carried out in closed Universal containers, and initiated by addition of triazene in 50 µl DMSO to experimental incubates, and also to control incubates containing liver inactivated by heating at 85-90°C for 15 minutes. Samples were taken at intervals and metabolism stopped by addition to an equal volume of ice-cold acetone and centrifuging for one minute in a Beckman microfuge B.

C.2.5.4 Metabolism and stability of monoalkyltriazenes and arylamines

Disappearance of substrate was compared in experimental incubates and in control incubates containing no liver, no cofactors, or heat-inactivated liver. Metabolism was started by addition of the substrate in 25 µl acetone, and samples were taken at time intervals, added to ice-cold acetone and centrifuged as described in section C.2.5.3.

Half-lives of monoalkyltriazenes were determined in Earl's buffer pH 7.4, also Tris and phosphate buffer at the same pH. The triazenes were incubated at a concentration of 100 µg ml^{-1} in closed Universal containers at 37°C. Samples were taken at intervals and added to an equal volume of ice-cold acetone to prevent further decomposition. The decrease in triazene concentration with time was assayed by HPLC.

C.2.5.5 Metabolism of AcMMT by TLX5 lymphoma ascites cells

Aliquots (1.5ml) of TLX5 and TLX5R cells were placed in closed Universal containers. AcMMT (5µg) in 25 µl DMSO was added to each incubate, both experimental and controls

containing TLX5 cells inactivated by heating at 90°C for 15 minutes or by sonic disruption for 30 seconds at 16 μ in a MSE ultrasonic disintegrator. Samples were taken at intervals during the 2 hour incubation at 37°C, and added to an equal volume of ice-cold acetone. After centrifugation, the decrease in AcMMT concentration was assayed by HPLC.

Cell viability at 2 hours was ascertained by the trypan blue dye exclusion test (section C.3.3).

C.2.6 In vivo metabolism

Female Bk/Wistar rats were housed individually for one day prior to drug administration in metabolism cages, and in the same cage throughout the experiment. AcDMT was administered i.p. to rats at a dose of 100 mg kg^{-1} dissolved in 10% acetone in arachis oil. Urine was collected in cooled containers and samples taken 6 and 24 hours after drug administration.

Blood was taken from animals dosed with AcDMT and anaesthetized using a mixture of halothane, nitrous oxide and oxygen, by cardiac puncture into a disposable syringe previously rinsed with 0.05 ml of heparin 2,500 U ml^{-1}. Plasma was obtained by centrifugation of the sample for one minute in an Eppendorf 5412 centrifuge (speed 7).

C.2.7 Preparation for assay

C.2.7.1 Samples for tlc

After addition of deproteinising agent to metabolic incubates, protein was sedimented by centrifugation for 5 minutes at maximum speed in a MSE bench centrifuge. The supernatant was extracted with 3 x 10 ml portions of either

dichloromethane or ether/methanol (70:30). Urine and plasma samples were extracted with dichloromethane. The extracts were dried with anhydrous magnesium sulphate, filtered and the volume reduced by vacuum distillation. The samples were applied to tlc plates which were then developed as described in section C.2.9 and Rf values were compared with those of reference compounds.

C.2.7.2 Samples for HPLC

C.2.7.2.1 Normal phase

After metabolic incubation, remaining substrate was extracted immediately without additional protein precipitation. AcDMT was extracted with ethyl acetate and aminopyrine with dichloromethane for 30 minutes on an Astell roll bottle apparatus. The extract layer was removed and in preliminary experiments dried with anhydrous magnesium sulphate; later this stage was found not be necessary. The solvent was concentrated by vacuum distillation or evaporated with a constant stream of nitrogen using a SC-3 sample concentrator with a Techne Dri-Block D8-3 kept to 30°C for efficient solvent removal without triazene decomposition.

C.2.7.2.2 Reverse phase

After centrifugation of the sample with acetone, it was kept in the freezer prior to HPLC assay. Sample injection was made as quickly as possible to minimize further breakdown of unstable triazene substrates and metabolites.

C.2.8 Nash colorimetric assay for formaldehyde

This assay first described by Nash (134) is based on the Hantzsh synthesis of pyridines (135) and employs acetylacetone which has been described as the most highly

selective reagent for the determination of formaldehyde (136). The method used in the present work was as described by Werringloer (137).

Metabolic supernatant after protein removal (2ml) was added to 1 ml of Nash reagent and heated for 20 minutes at 60°C. The resulting yellow colour was quantitated by absorbance readings at 412 nm using a Cecil CE 5095 spectrophotometer. A standard formaldehyde curve was produced using paraformaldehyde. Formaldehyde was liberated upon addition of 1 ml 1N sodium hydroxide solution, and the formaldehyde solution made up to volume in Earl's buffer. Dilutions were made to prepare formaldehyde concentrations of 0-200 μM, deproteinising agent was added as in the metabolic samples, and absorbance readings obtained with Nash reagent (see Appendix).

C.2.9 tlc assay

Initially glass tlc plates were spread using either silica or alumina absorbants. Later prepared silica plates containing a 254 nm fluorescent indicator were used. Spray reagents aided identification of metabolites: NEDA to identify an intact triazene linkage (purple) and p-dimethylaminobenzaldehyde to detect aromatic amines (yellow). For routine use, prepared silica plates and solvent systems of toluene/ether (1:9) or toluene/acetone (4:1) were employed.

C.2.10 HPLC assay

C.2.10.1 Normal phase

This system was used for metabolism experiments measuring disappearance of AcDMT and aminopyrine. Samples were redissolved in the HPLC mobile phase and injected on to

the column using a 100 µl loop. All injections were made in duplicate, and quantification was by measurement of peak height. Extraction efficiency was determined by comparison with the peak height obtained for dilutions of the substrate stock solution used for the metabolic incubations.

A silica column was used - Partisil PXS 10/25, 25 cm x 46 mm (Whatman). The mobile phase was chloroform/dichloromethane (20:80) for AcDMT; and for aminopyrine with addition of 3% methanol. An Altex 110A pump provided the mobile phase flow rate of 2ml/min. Detection by a Cecil Instruments variable wavelength detector was at the λ max of the compound - 337 nm for AcDMT and 260 nm for aminopyrine.

C.2.10.2 Reverse phase

HPLC columns, flow rates and solvent systems for the various determinations are shown in table 5. A cyano 10 µ radial pak liquid chromatography cartridge (Waters), or a Spherisorb 5µ ODS 25 cm x 46 mm column were used. Chromatography was performed using an Altex 100A pump in conjunction with a Pye-Unicam variable wavelength LC-UV detector. Deproteinised metabolic incubates were injected directly on to the column using a 20 µl loop and an Altex rheodyne injector, and quantification was made by measurement of peak height.

C.2.10.3 Calibration curves

Reverse phase HPLC calibration curves were constructed for all metabolites likely to be produced on dialkyltriazene metabolism (see section C.2.5.3). Concentrations in the ranges 0-50 or 0-100 µg ml^{-1} of each of the compounds were

made up in 2.5 ml volumes of ice-cold Earl's buffer by addition of not more than 50 µl stock solution in DMSO. Samples were taken immediately and added to an equal volume of ice-cold acetone. Calibration curves were obtained from at least duplicate series of dilutions, with peak height measurements taken at the same wavelength and under the same HPLC conditions as for the metabolism assays (see Appendix).

Table 5 Reverse phase HPLC conditions for triazene assay

Metabolic substrate	Column	Flow Rate (ml/min)	Mobile phase (% aqueous acetonitrile + 0.1% diethylamine)	UV detection (nm)
AcDMT	CN	2	35	325
AcMMT	ODS or CN	2	35	325
AcDET	CN	2	35	325
AcMET	CN	2	35	325
CyDMT	CN	2	30	300
CyMMT	CN	2	30	300
CyDET	CN	2	30	300
CyMET	CN	2	30	300
(Iu)	CN	2	30	300
(Iv)	CN	3	35	300
(Idd)	ODS	2	32	340

C.3 Toxicity studies

C.3.1 Hepatocyte preparation

Preparation was made by an adaptation of the method of Renton et al (138).

C.3.1.1 Solutions

a) Calcium free Hepes buffer pH 7.4.

b) Hepes/EGTA - calcium free Hepes buffer containing EGTA 490 mg L^{-1}.

c) Collagenase perfusion medium - 50 ml calcium free Hepes buffer containing 0.5 ml calcium chloride solution (170 mg ml^{-1}) and 25 mg collagenase type IV.

d) Incubation medium - Krebs Henseleit buffer pH 7.4 containing 10% horse serum and 1% BSA.

All media were gassed with 95% O_2/5% CO_2 (BOC) for several minutes before and throughout the perfusion, and were kept at a temperature of $37^{o}C$.

C.3.1.2 Perfusion procedure

A male CBA/Ca mouse was anaesthetized by i.p. injection of 0.25 ml Sagatal (15 mg ml^{-1} in sterile saline). The inferior vena cava was cannulated in a retrograde fashion by the procedure of Renton et al (138). During cannulation, Hepes/EGTA was pumped at 7ml/min (LKB 2132 Microperplex peristaltic pump) and the liver was seen to clear immediately and drain through the portal vein. Upon cannulation, flow was switched to calcium-free Hepes buffer for 2 minutes via a 3-way junction. The liver was then perfused with all of the collagenase medium and flow switched back to calcium-free Hepes

buffer for 1 minute in excess of the lag time of the apparatus in order to flush the collagenase from the liver.

After this time, the liver was carefully excised and placed in a petri dish containing Hepes buffer. The liver was gently broken apart with forceps and the cells released. Freed cells were poured through nylon mesh into a beaker. Fresh buffer was added to the tissue, and the procedure repeated several times until a sufficient number of cells had been isolated.

The pooled fluid was gently swirled to suspend the cells and poured into centrifuge bottles. The cells were either allowed to settle while placed on ice, or centrifuged for 4 minutes at 50g in a MSE bench centrifuge. The supernatant was removed and the cells resuspended in Krebs incubation medium. After recentrifugation and resuspension in about 5ml incubation medium, the cells were kept on ice until used.

C.3.1.3 Assessment of viability

A 1 in 10 dilution of the suspended hepatocytes was made in trypan blue solution (0.3% in Krebs Henseleit buffer). After mixing, a total cell count was made on a haemacytometer (Weber) at X10 magnification. Cells excluding the dye were considered viable whereas those stained blue were considered non-viable. The former group were calculated as a percentage of the total cell count as an expression of cell viability.

C.3.2 Toxicity of AcDMT and AcMMT to mouse hepatocytes

The hepatocytes prepared as described in section C.3.1 were suspended at 2×10^6 cells per ml in Krebs

incubation medium and 5ml aliquots were placed in 25ml Quickfit conical flasks. These flasks, and all other glassware coming into direct contact with the hepatocytes had previously been treated with a silanising agent, dimethyldichlorosilane, to prevent cell adhesion to the glass surface. A range of concentrations of AcDMT and AcMMT were added in up to 50 µl DMSO, with control incubations receiving DMSO only. The flasks were incubated in a water bath for 2 hours at 37°C, shaking at minimum speed. At all times, the flask atmosphere was saturated with 95% O_2/5% CO_2.

Samples (1ml) were taken from each of the flasks at 1 and 2 hours. After briefly allowing the hepatocytes to settle, a 50 µl sample was diluted 1 in 10 with trypan blue solution, and viability assessed as described in section C.3.1.3. Results were expressed as percentage increase in trypan blue staining in experimental over control incubates.

C.3.3 Toxicity of AcDMT and AcMMT to TLX5 ascites cells

Both triazene sensitive and resistant TLX5 cells were harvested as in section C.2.4 but suspended in Krebs incubation medium at 2 x 10^6 cells per ml. Aliquots (2ml) were incubated for 2 hours in closed Universal tubes at 37°C in a shaking water bath. A range of concentrations of triazene was added at time 0 in 50 µl DMSO; control incubations received DMSO only.

After 2 hours, a 1 in 5 dilution of the cells was made in trypan blue solution, and cell viability assessed by

cell counts at x40 magnification. A percentage increase in trypan blue staining in experimental over control cells was determined.

C.4 Glutathione studies

C.4.1 Assay for glutathione

The glutathione assay method used is specific for GSH and GSSG, unlike early methods employing 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (139) which assayed all sulphydryl compounds. The method is an adaptation of that of Griffith (140), which is itself based on the enzymatic recycling assay of Tietze (141).

C.4.1.1 Solutions

(a) Phosphate - EDTA buffer pH 7.5.

(b) 10% metaphosphoric acid (stored in the refrigerator). (4°C)

(c) Trisodium phosphate prepared freshly 12.5 mg in 25 ml distilled water, and with constant stirring and heating to keep in solution.

(d) DTNB 2.38 mg ml^{-1} phosphate - EDTA buffer (stored in the freezer protected from the light).

(e) NADPH 0.3 mM in phosphate-EDTA buffer.

(f) Glutathione reductase 50 IU ml^{-1} in phosphate-EDTA buffer.

(g) 2-Vinylpyridine.

C.4.1.2 Enzyme assay

(300µl)
Test sample in 10% metaphosphoric acid was pipetted into each of two microfuge tubes. To each was added 120 µl of the trisodium phosphate solution to neutralise the acid, and to one was added 6 µl 2-vinylpyridine. Both tubes were sealed, mixed, and incubated in a shaking water bath at 30°C for 20 minutes. During this time, the 2-vinylpyridine reacts with the thiol group of the reduced glutathione

(GSH), but not of oxidised glutathione (GSSG). After incubation, the solutions were microfuged for 2 minutes. Supernatant (140 µl) was pipetted into a 1 ml capacity quartz cuvette and to this was added 100 µl DTNB solution, 700 µl NADPH solution and 10 µl glutathione reductase. After thorough mixing, the cuvette was placed in a holder maintained at 30°C in a Cecil Instruments CE5095 spectrophotometer, and the change in absorbance at 412 nm was measured against a reference cuvette containing 100 µl DTNB solution, 100 µl 10% metaphosphoric acid, 40 µl trisodium phosphate solution and buffer to volume. The rate of colour formation due to DTNB reduction is dependent on the glutathione content of the sample, and may be calculated from the slope of a fixed wavelength scan (Cecil Instruments CE500 control-record module). A blank solution containing 700 µl NADPH, 100 µl DTNB, 100 µl acid, 40 µl base and 10 µl glutathione reductase was assayed to take into account the background reduction of DTNB by the NADPH-glutathione reductase system alone without the presence of glutathione. Glutathione was evaluated by comparison of the result with a standard curve; the concentration in the vinylpyridine containing tube indicates GSSG levels in the sample, and the concentration in the other tube the total glutathione content.

C.4.1.3 Calibration curve

A series of concentrations of GSH and GSSG were made up in 10% metaphosphoric acid. These were assayed as described in section C.4.1.2 with and without vinylpyridine in order to construct a total glutathione calibration curve

expressed as GSH + $\frac{1}{2}$ GSSG (see Appendix). A standard GSH solution was assayed together with test samples to check the functioning of the enzyme assay.

C.4.2 Glutathione levels in TLX5 ascites cells

Both TLX5 and TLX5R cells were harvested as described in section C.2.3, and suspended in Krebs incubation medium at 2 x 10^6 cells per ml. Samples (5ml) were taken and centrifuged for 2 minutes at speed 2 in a MSE bench centrifuge. The supernatant was removed and the cells resuspended in 700 µl 10% metaphosphoric acid. Samples (300µl) were taken for duplicate assays of total glutathione as described in section C.4.1.2.

C.4.3 Assay of glutathione in the presence of triazenes

A 50 µM solution of GSH in 10% metaphosphoric acid was prepared and 2.5 ml aliquots placed in 25 ml flasks. To the control was added 50 µl DMSO or no solvent, and to the other flasks varying concentrations of AcDMT and AcMMT in 50 µl DMSO. Duplicate 300 µl samples were taken immediately from each of the flasks and assayed for total glutathione and GSSG as described in section C.4.1.2.

C.4.4 Glutathione levels in mouse hepatocytes

Aliquots (2.5ml) of hepatocytes in Krebs incubation medium 2 x 10^6 per ml were pipetted into 25 ml Quickfit flasks. To the control flask was added 50 µl DMSO, to the others a range of concentrations of AcMMT in 50 µl DMSO. Incubation was at 37^{0}C in a water bath shaking at minimum speed and with constant gassing with 95% O_2/5% CO_2. Samples were taken at 0, 40 and 80 minutes. At each time point, the contents of a

flask were used (5 million cells) because of the difficulty of sampling as hepatocytes settle very quickly, and also the large number of cells was required for sufficient glutathione for assay. Viability of the hepatocytes, as assessed by the trypan blue exclusion test (section C.3.1.3) was determined at time 0 and 80 minutes.

The 2.5 ml hepatocyte samples were poured into centrifuge tubes and centrifuged at 50g in a MSE bench centrifuge. The supernatant (0.5ml) was added to 0.5 ml of 20% metaphosphoric acid in a microfuge tube. After discarding the remaining supernatant, the cells were suspended in 1 ml 10% metaphosphoric acid. This cell suspension was transferred to a microfuge tube and all samples were microfuged for 2 minutes. The supernatants were used in the glutathione assay as described in section C.4.1.2.

C.4.5 Glutathione reductase assay

Glutathione reductase was assayed by the method of Worthington and Rosemeyer (142).

C.4.5.1 Solutions

a) Potassium phosphate buffer with EDTA and KCl pH 7.0.

b) GSSG 10mM in buffer.

c) NADPH 1mM in buffer.

C.4.5.2 Enzyme assay

Assays were carried out at 25°C in the thermostatically controlled cell holder of a Cecil Instruments CE5095 spectrophotometer. The reference cuvette contained buffer only, and to the sample cuvette was added 700 µl buffer, 100 µl NADPH solution, 100 µl GSSG solution and

100 µl of the test sample. A change in absorbance at 340 nm was recorded; the slope of a fixed wavelength scan is proportional to the rate of oxidation of NADPH, and hence the glutathione reductase level in the sample. A blank solution containing buffer, NADPH and GSSG but no reductase was assayed to account for autoxidation of NADPH under these conditions.

C.4.6 Glutathione reductase in mouse hepatocytes

Incubations (5ml) of mouse hepatocytes in Krebs incubation medium 2 x 10^6 cells per ml were used. Various concentrations of AcDMT and AcMMT were added in 50 µl DMSO; control incubations contained DMSO only. To a positive control was added 50 µM BCNU, a known inhibitor of glutathione reductase (143), in order to test the assay procedure. Incubation was at 37°C in a water bath shaking at minimum speed, and with constant gassing with 95% O_2/5% CO_2. Samples (1ml) were taken from flasks at 0, 40 and 80 minutes with gentle swirling to suspend the cells. Viability of the hepatocytes was assessed by trypan blue exclusion at the beginning and end of the experiment (section C.3.1.3).

The 1 ml samples were centrifuged at 50g in a MSE bench centrifuge. The supernatant was discarded and 0.5ml of potassium phosphate/EDTA/KCl buffer was added to resuspend the cells. The cells were disrupted by sonication for 20 seconds at 16 µ in a MSE ultrasonic disintegrator in order to release the enzyme. The resulting suspensions were centrifuged for 2 minutes in a Beckman microfuge B. The supernatant was used in the assay as described in section C.4.5.2.

C.4.7 Glutathione oxidation in buffer and mouse liver homogenate

C.4.7.1 Preparation of mouse liver homogenate

Livers were excised from male CBA/Ca mice and washed in 0.25M sucrose. A 25% $^{w}/_{v}$ homogenate was prepared in further ice-cold sucrose using 8 strokes of a Camlab 563C homogeniser (speed 5) fitted with a teflon pestle. The resulting homogenate was centrifuged for 5 minutes at speed 5 in a Heraeus Labofuge 6000 to remove cell walls and debris. The supernatant was used in incubations as described in section C.4.7.2.

C.4.7.2 Incubation conditions

The basic liver incubation was made up of 0.6 ml of homogenised liver supernatant and 1.9 ml Earl's buffer pH 7.4 in a 25 ml pyrex beaker. This was incubated in a shaking water bath (80 strokes/min) at 37°C for 20 or 30 minutes. Samples (0.4ml) were taken at time 0 and at 10 minute intervals and added to an equal volume of 20% meta-phosphoric acid in a microfuge tube. After microfuging for 2 minutes, the sample was stored on ice until used in the glutathione assay as described in section C.4.1.2.

Variations on the basic incubation were to incubate on ice rather than at 37°C, under a nitrogen atmosphere rather than air, and in sucrose rather than Earl's buffer. Further incubations contained liver homogenate previously heated to 90°C for 15 minutes in order to inactivate enzyme systems. Another variation was to incubate GSH made up in Earl's buffer (150 μM) rather than endogenous liver glutathione.

For all samples, both total glutathione and GSSG were determined, and the GSSG was expressed as a percentage of the total glutathione, and its increase with time observed.

C.4.7.3 Perfused mouse liver

A male CBA/Ca mouse was anaesthetized and cannulated as described in section C.3.1.2. In this study, the perfusion fluid was cold 0.25M sucrose and perfusion was continued for 1 minute until the liver was cleared of blood. The liver was then excised and a liver homogenate prepared as described in section C.4.7.1. Incubations were made in Earl's buffer at 37°C in the same way as described for untreated liver (section C.4.7.2).

C.4.7.4 Drug additions to incubations

Monomethyltriazenes, monoethyltriazenes and arylamines were added to the basic liver incubate in 25 µl acetone at time 0 to study the effect on the rate of GSH oxidation. Control incubations received 25 µl acetone only.

EDTA was added to incubates at different concentrations dissolved in Earl's buffer, to determine the rôle of metal ions in the oxidation of GSH under these conditions. Diethyldithiocarbamic acid (1mM and 10mM) and sodium azide (1mM) were added to incubations dissolved in Earl's buffer.

PART D

RESULTS

PART D RESULTS

D.1 Stability studies

D.1.1 Photodecomposition of DTIC

Photodecomposition of DTIC (IIa), and decomposition of diazo-IC (V) in the presence and absence of sunlight were followed at a range of pH values by UV-visible spectrometry. The products obtained were identified by comparison with reference spectra recorded at the appropriate pH. 2AH (IV) and other photoproducts are amphoteric and the wavelength of maximum absorption varies with pH (table 6). The results are summarized in table 7.

Control experiments confirmed the stability of 2AH to light, and therefore this cyclic product is not an intermediate in the production of 4-carbamoylimidazolium-5-olate (OIC, IXa)- also a stable imidazole.

Bright sunlight did not qualitatively effect the decomposition pathway, but accelerated decomposition as compared with diffuse natural light.

Diazo-IC was shown to decompose in the dark: under these conditions the product was always 2AH (figure 8). In the presence of sunlight, photoproducts as for DTIC were identified (table 7), with the production of OIC in the pH range 2 - 6 (figure 9).

DTIC proved to be most stable under mildly acidic conditions. At pH3, photodecomposition was complete at room temperature ($\sim$ 20^{o}C) in diffuse sunlight within 2 hours, but at pH 7.4 and above, the drug had completely decomposed within the first 15 minute time interval. Diazo-IC was similarly most stable at pH3, in the dark (table 8).

Table 6 Variation of λ max (nm) of imidazole derivatives with pH.

pH	diazo-IC (V)	2AH (IV)	OIC (IXa)	AIC (III)
1	237,293	250,277	236,275	239,264
2.5	246,312	250,277	236,276	241,263
7.4	*245,310	250,288	236,276	*230,265
10.15	immediately cyclizes to 2AH	254,294	236,276	*230,265

* inflexion

Table 7 Products formed on decomposition of DTIC and diazo-IC at different pH values.
(substrate concentration ~ 0.01 mg ml^{-1})

Substrate/pH		1	2	3	5.2	7.4	8.5	10.15	12
DTIC-Dome	A	IV	IXa	IXa	IXa	IV	IV	IV	IV
	B	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa
DTIC(pure) +	A	IV	IXa	IXa	IXa	IV	IV	IV	IV
	B	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa
Diazo-IC *	A	IV	IXa	IXa	IXa	IV	IV	IV	IV
	B	IV	IV	IV	IV	IV	IV	IV	IV

A - sunlight

B - in the dark

* - sample dissolved directly in buffer

+ - sample dissolved in DMSO and diluted with buffer prior to photolysis

Table 8 Half-life of diazo-IC in the dark at 37°C under different pH conditions

pH	$t_{\frac{1}{2}}$(min)
1	4.9
2	19.3
3	34.6
5.2	22.2
7.4	< 2

Photolysis of more clinically realistic concentrations of DTIC-Dome (1 mg ml^{-1}) resulted in effervescence and formation of a maroon precipitate. After removal of the precipitate, UV spectroscopy revealed the presence of 2AH in the straw-coloured filtrate. The pH remained stable throughout the decomposition, and was not raised by the dimethylamine liberated.

D.1.2 Interaction between DTIC and nucleophiles in the presence of sunlight

In order to search for chemical interaction between DTIC and other compounds, the decomposition of DTIC or diazo-IC with the other agents in solution was monitored at mildly acidic pH. Any alteration in the triazene decomposition pathway, with the resultant appearance of a new peak in the UV-spectrum, would be indicative of a chemical interaction.

D.1.2.1 Thiol compounds

An interaction was seen with the thiol groups of L-cysteine, glutathione and 2-mercaptoethanol. The S-methyl group of methionine did not interact.

A spectroscopically identical product was formed upon photolysis of DTIC, or decomposition of diazo-IC in the presence of L-cysteine and was characterized by a peak of λ max 340 nm. The product was unstable as shown by the disappearance of this peak with time in both the dark and the light.

D.1.2.2 Other cytotoxic drugs

A series of cytotoxic drugs (table 9) was used in the study to investigate possible interactions with DTIC in the presence of sunlight. In no case was there any interaction as detected by UV spectroscopy.

Table 9 Cytotoxic drugs used in the interaction study with DTIC

Alkylating agents	cyclophosphamide
	melphalan
	thiotepa
Antimetabolites	cytosine arabinoside
	5-fluorouracil
	6-mercaptopurine
	methotrexate
Plant alkaloids	vinblastine
Nitrosoureas	BCNU
	CCNU
Anthracycline antibiotics	daunorubicin
	doxorubicin
Other antibiotics	bleomycin
	dactinomycin

D.1.2.3 Formation of azo-dyes

Photodecomposition of DTIC in the presence of N-(naphth-1-yl)ethylenediamine (NEDA), or a mixture of diazo-IC with NEDA yielded an unstable product with a peak of λ max 512nm, and an intense purple colouration, 2-naphthol was found to be less reactive, but a mixture of diazo-IC and 2-naphthol yielded an unstable product characterized by a peak of λ max 496nm, and a scarlet red colouration.

D.1.3 Stability of 1-(4-acetylphenyl)-3,3-dimethyltriazene (AcDMT) (Ij)

The stability of AcDMT was determined at different pH values at 37°C in the dark. The decrease in absorption was measured at the λ max of the compound, 337nm, by UV spectroscopy. The results are shown in table 10.

D.1.4 Stability of monomethyl- and hydroxymethyl- triazenes

The stability of several derivatives was determined at physiological pH (7.4) in phosphate buffer at 37°C in the dark by UV spectroscopy.

D.1.4.1 5-(3-Methyltriazen-1-yl)imidazole-4-carboxamide (MTIC)

MTIC was first dissolved in methanol, in which it has a half-life of 8 hours (13), and 1 - 2 drops of this solution were added to the cuvette of buffer for the stability determination. MTIC decomposed even in the dark to yield AIC (figure 10), as identified by comparison with the spectrum of the authentic compound. The calculated half-life was 2.35 minutes at 37°C.

Table 10 Half-life of AcDMT in the dark at 37°C under different pH conditions.

pH	$t_{\frac{1}{2}}$
2.5	1.2 min
3.5	11.8 min
4.5	107.7 min
5.2	> 24 hours *
7.4	> 24 hours *

* Also stable in the light

D.1.4.2 Aryltriazene derivatives

Half-lives of two different para- substituted monomethyltriazenes (AcMMT; Iy) and hydroxymethyltriazenes (Il;Iz) were determined as described previously by UV spectroscopy. Phosphate buffer pH 7.4 was used, as in all other UV stability studies at this pH. For comparison, the half-life of AcMMT was determined at 37°C in three different buffers of pH 7.4 by an HPLC method.

The half-life of a third monomethyltriazene (Ibb) was calculated from the decrease in absorbance of a fixed wavelength scan (at 300nm) with time on a Beckman Acta V spectrophotometer. In each case, the product of decomposition was the corresponding arylamine. The results are shown in table 11.

Table 11 Half-lives of monomethyl- and hydroxymethyl-triazenes in the dark at 37°C in different buffers of pH 7.4.

Compound	Buffer (pH 7.4)	max (nm)	$t_{\frac{1}{2}}$ (min)
AcMMT (Ik)	phosphate (uv)	325	13.8
AcMMT (Ik)	phosphate (HPLC)	325	15.1
AcMMT (Ik)	Tris	325	36.2
AcMMT (Ik)	Earl's	325	66.6
(Il)	phosphate	325	14.5
(Iy)	phosphate	313	12.1
(Iz)	phosphate	313	12.6
(Ibb)	phosphate	300	1.2

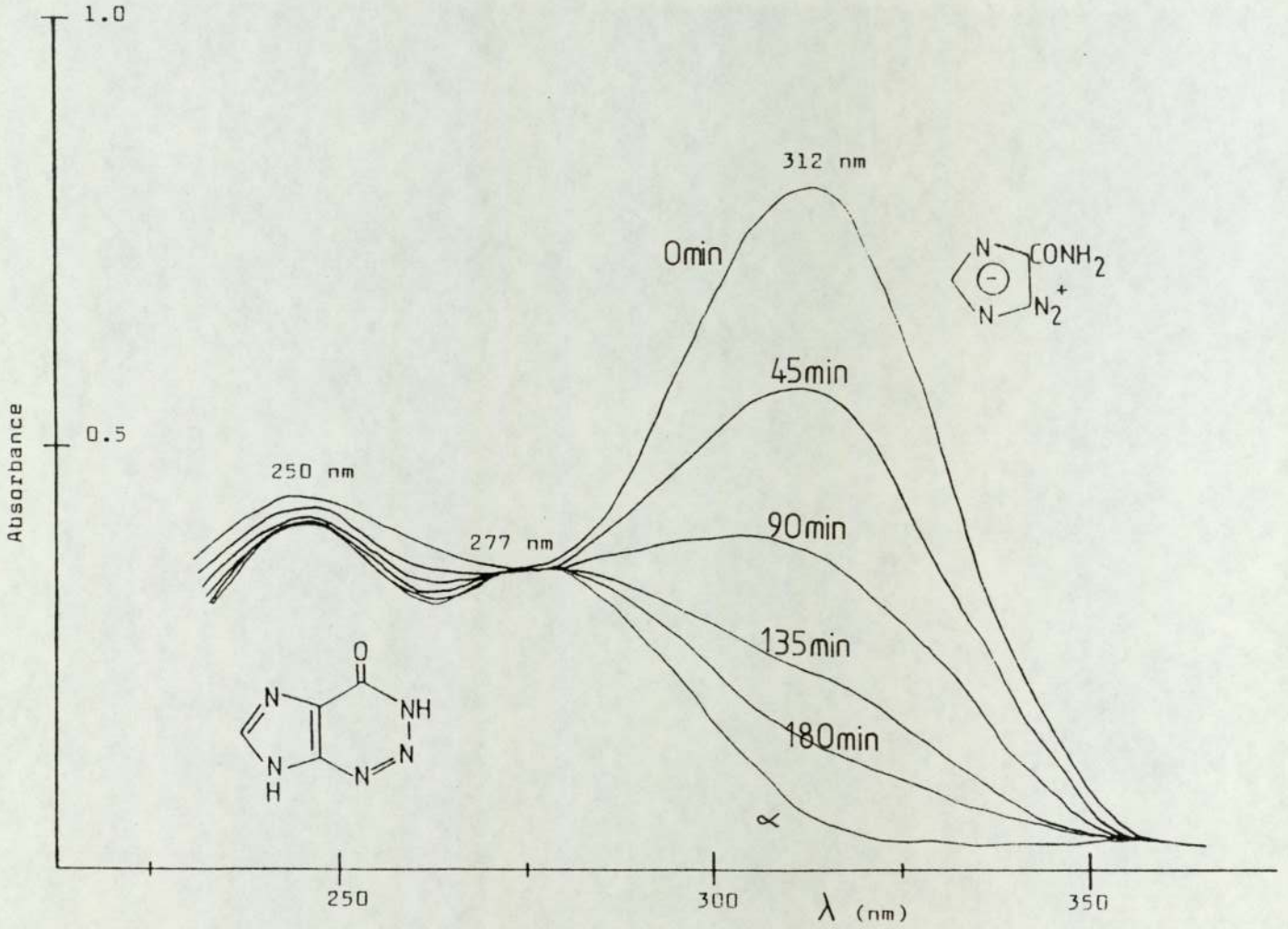


Figure 8 Decomposition of diazo-IC at pH 2.5 in the dark.

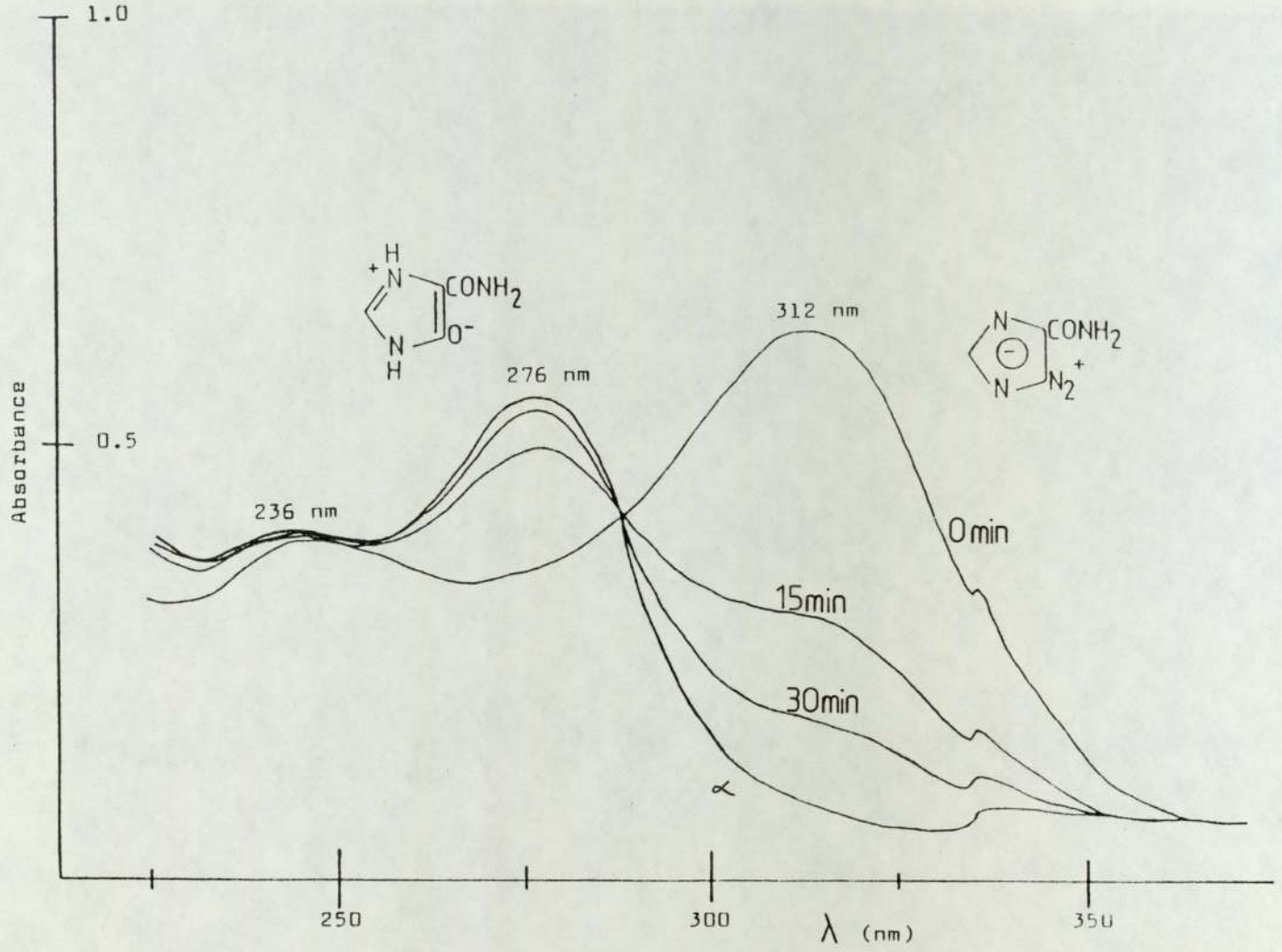


Figure 9 Decomposition of diazo-IC at pH 2.5 in the presence of sunlight.

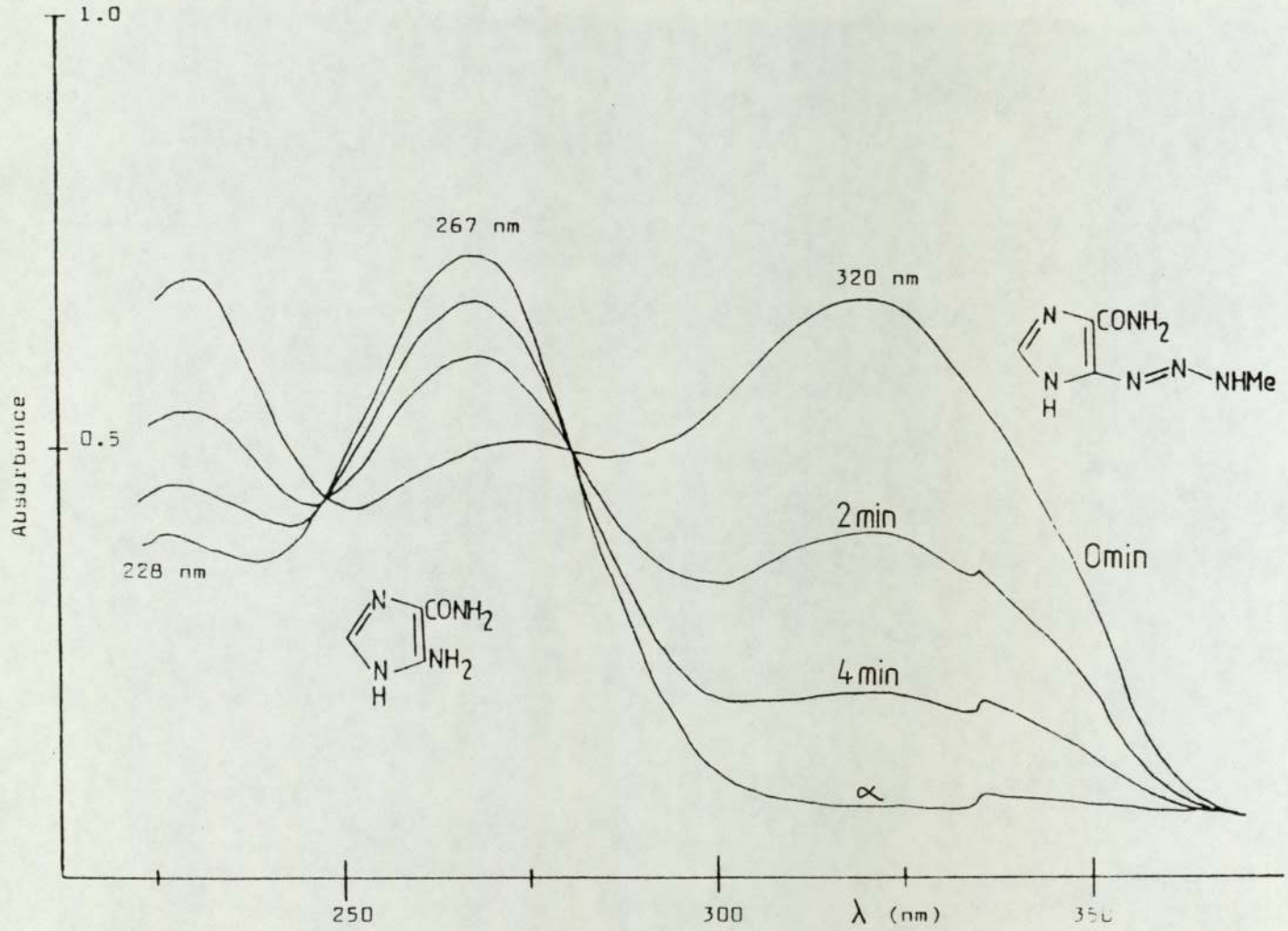


Figure 10 Decomposition of MTIC at pH 7.4 in the dark.

D.2 Metabolism studies

D.2.1 Preliminary investigations on the metabolism of 1-(4-acetylphenyl)-3,3-dimethyltriazene (AcDMT)(Ij).

D.2.1.1 Development of a tlc system

Both *in vitro* and *in vivo* metabolism of AcDMT were studied. Tentative identification of metabolites was made by tlc as described in section C.2.9. Typical Rf values of AcDMT and its possible metabolites are given in table 12 for a prepared silica plate and a solvent system toluene/acetone (4:1). Spray reagents were used as an aid to metabolite identification, and the colours obtained for each of the compounds are also shown in table 12.

D.2.1.2 In vitro and in vivo metabolism of AcDMT

In vitro metabolism of AcDMT by rat liver 9000g fraction was carried out in parallel with control incubations containing buffered sucrose or liver, but no cofactors, for 30 minutes. For the *in vivo* studies, urine was collected from rats 6 and 24 hours after injection of the triazene (100 mg kg^{-1}), or blood samples were taken at 2, 3 and 4 hours. The metabolic products identified are shown in table 13.

D.2.2 Metabolism of 1-(4-acetylphenyl)-3,3-dimethyltriazene (AcDMT)(Ij) under the conditions of the bioassay and comparison with aminopyrine (XIa)

D.2.2.1 HPLC assay

Disappearance of substrate with time was measured by normal phase HPLC as described in section C.2.10.1.

The separation of standard solutions of AcDMT and 4-aminoacetophenone (Xa) is shown in figure 11. The

Table 12 Rf values and spray reagent characterization of AcDMT and some possible metabolites.

Compound	Rf	Colour with spray reagent	
		N-(Naphth-1-yl) ethylenediamine	4-Dimethyl-aminobenzaldehyde
AcDMT	0.82	dark purple	pale yellow
AcMMT	0.76, 0.68, 0.59Ø	dark purple	yellow, turning orange with time
(I1)	0.76-0.55* (0.39)+	dark purple	yellow, turning orange with time
(Xa)	0.58	pale yellow	orange yellow
(VIII)	0.67	pinkish purple	-
Spray reagent alone		colourless	pale yellow

Ø separates as three spots

* streak

\+ spot observed in high concentration samples only

Table 13 tlc identification of *in vitro* and *in vivo* metabolites of AcDMT

In vitro		In vivo
Control	experimental	(plasma and urine)
AcDMT	AcDMT	AcDMT
	(Xa)	(Xa)
	(VIII)	
	more polar metabolites	

monomethyltriazene (AcMMT, Ik) and the hydroxymethyltriazene (Il) are unstable and cannot be assayed by this method. Their retention times were between those of AcDMT and the arylamine (Xa). Calibration curves for AcDMT showed that the detector response as measured by peak height increased linearly with increased triazene concentration in the range used in these experiments. The HPLC assay for aminopyrine was similar to that used for the triazene (section C.2.10.1)

Extraction efficiencies for AcDMT and aminopyrine during preparation of metabolic samples for assay (section C.2.7.2.1) are shown in table 14.

D.2.2.2 Metabolism of AcDMT

A substrate concentration of 50 μM AcDMT was used in the metabolic incubates, since a measurable level of metabolism, as assayed by substrate disappearance, could be observed over the two hour incubation. AcDMT was found to be stable in control incubations containing liver preparations without cofactors, or liver inactivated by heating.

Metabolism of AcDMT by the 9000g fraction of liver from male CBA/Lac mice is shown in figure 12. Incubations were either allowed free access to air (open system), or were carried out in closed 20 ml Universal tubes (closed system). Figure 13 shows results from a similar experiment using the 9000g fraction prepared from female BK/Wistar rats.

In table 15, a comparison is made between 9000g and microsomal metabolism of AcDMT in the closed system. The extent of metabolism by the 9000g fraction is expressed as a percentage of the microsomal value, the mean values for both liver preparations being used in the determination. Metabolism

Table 14 Extraction efficiencies for AcDMT and aminopyrine

Compound	Extraction solvent	Extraction efficiency
AcDMT	ethylacetate	82.8 ± 3.7
aminopyrine	dichloromethane	72.4 ± 2.0

Table 15 Comparison of 9000g and microsomal metabolism of AcDMT (50 µM) in the closed system

Animal	9000g metabolism as % of microsomal metabolism		
	15 min	30 min	2 hours
female rat	100	102.5	103.1
female CBA/Lac mouse	70.5	87.7	86.6
male CBA/Lac mouse	84.7	82.0	89.0

of AcDMT (50 μM) in the closed system using male and female CBA/Lac mouse 9000g and microsomal preparations is compared in table 16.

Figure 14 shows a comparison between rat and mouse microsomal metabolism of AcDMT in the closed system. Similar results were seen using 9000g preparations.

The metabolism of AcDMT was also studied in liver preparations from rats pretreated with phenobarbitone. Metabolism in both 9000g and microsomal fractions was induced to the same extent. The induction of microsomal metabolism in the closed system is shown in figure 15.

D.2.2.3 Metabolism of aminopyrine

Initial determinations of aminopyrine metabolism were made by measuring the formaldehyde and formaldehyde precursors produced during metabolism of the drug for 30 minutes by the Nash colorimetric assay (section C.2.8). A substrate concentration of 4mM was necessary in order to get a detectable level of species giving a positive test for formaldehyde. Further experiments were undertaken assaying aminopyrine metabolism by substrate disappearance (by the HPLC method) after 30 minute incubations and for these a substrate concentration of 50 μm was used as in the AcDMT study.

Results using liver preparations of female CBA/Lac mice and female rats for the metabolism of aminopyrine (4mM) measured by the Nash assay for formaldehyde are shown in figure 16 for the open system. The rate of metabolism was significantly lower ($P = 0.0001$) using rat rather than mouse

Table 16 Comparison of metabolism of AcDMT (50 μM) by preparations from male and female CBA/Lac mice in the closed system

Time	% AcDMT metabolised			
	9000g		microsomes	
	male	female	male	female
15 min	19.5± 1.8	21.7± 8.1	23.0± 7.0	30.8± 11.9
30 min	30.1± 4.5	40.5± 9.9	36.7± 8.8	46.2± 6.3
2 hours	63.3± 4.4	73.0± 6.2	71.1± 17.0	84.3± 10.4

microsomal preparations. Figure 17 shows the results of similar experiments, however here the microsomal metabolism of aminopyrine (50 μM) was assayed by substrate disappearance as measured by HPLC. A significant difference was again seen ($P = 0.0001$) between the microsomal preparations from the two species. Rat/mouse comparisons of the different incubation conditions and assay methods for microsomal metabolism of aminopyrine are shown in table 17.

Male and female CBA/Lac mouse microsomal metabolism of aminopyrine (4mM) as detected by the Nash assay are compared in table 18. In the open system, there was a significant difference between preparations from male and female animals ($P < 0.05$).

Figure 18 shows the varying concentrations of species giving a positive test in the Nash assay yielded on metabolism of aminopyrine, in the open system, with the use of different liver preparations from male CBA/Lac mice. A similar trend was seen for rat liver preparations. A significant difference was seen between the extent of metabolism of aminopyrine in female CBA/Lac mouse 9000g and microsomal preparations using both the colorimetric and chromatographic assay methods (figures 16 and 19). A significant difference was not found, however, using liver preparations from another strain of female mouse (CBA/Ca), measuring metabolism by substrate disappearance (table 19).

The effect of pretreating rats with phenobarbitone on the extent of microsomal metabolism of aminopyrine is shown in table 20.

Table 17 Comparison of aminopyrine metabolism by female CBA/Lac mouse and female rat microsomal preparations

Incubation system	Aminopyrine concentration	Assay method	Rat metabolism as % of mouse metabolism
closed	4mM	Nash	37.3 (n=2)
open	4mM	Nash	35.5 *
open	50 μM	HPLC	18.6 *

* significant difference between rat and mouse preparations ($P = 0.0001$)

Table 18 Comparison of aminopyrine (4mM) metabolism by male and female CBA/Lac mouse microsomal preparations in open and closed systems

Incubation system	nmol formaldehyde and formaldehyde precursors formed per 100mg liver in 30 minutes	
	male	female
closed	37.6 ± 5.6	47.2 ± 10.4
open	48.8 ± 4.0*	60.8 ± 9.6*

* $P < 0.05$

Table 19 Comparison of aminopyrine metabolism by female mouse 9000g and microsomal preparations in the open system

Assay	Aminopyrine concentration	Strain of mouse	9000g metabolism as % of microsomal metabolism
Nash	4mM	CBA/Lac	18.5*
HPLC	50 μM	CBA/Lac	49.0*
HPLC	50 μM	CBA/Ca	76.7

* significant difference between 9000g and microsomal metabolism

Table 20 Comparison of aminopyrine metabolism by microsomal preparations from non-induced and phenobarbitone-induced female rat in the open system

Assay method	Aminopyrine concentration	Induced-rat microsomal metabolism as % of non-induced rat metabolism
Nash	4mM	299
HPLC	50 μm	327

The extent of metabolism was in each experiment expressed per 100 mg wet weight of liver. In order to ascertain whether species and sex differences in metabolism were due to variations in liver protein content, protein determinations were made on microsomal preparations by the method of Lowry (section C.2.3). The results are shown in table 21.

D.2.3 Quantification of products formed during metabolism of 4-acetyl- and 4-cyano-dialkyltriazenes

Metabolism of the six dialkyltriazenes studied in the present work was measured by a reverse phase HPLC assay, and metabolites were identified by co-chromatography with known authentic standards added to the metabolism samples. The derivatives used are listed below.

(Ij) 1-(4-acetylphenyl)-3,3-dimethyltriazene AcDMT

(Im) 1-(4-acetylphenyl)-3,3-diethyltriazene AcDET

(Iq) 1-(4-cyanophenyl)-3,3-dimethyltriazene CyDMT

(Is) 1-(4-cyanophenyl)-3,3-diethyltriazene CyDET

(Iu) 1-(4-cyanophenyl)-3-methyl-3-ethyltriazene

(Iv) 1-(4-cyanophenyl)-3-methyl-3-tert-butyltriazene

The HPLC systems used are described in section C.2.10.2, and the separations achieved for standard solutions of the dialkyltriazenes and their potential metabolites are shown in figures 20-25. The concentrations of the various metabolites produced over the two hour incubations were calculated from previously determined calibration curves (see Appendix).

The dialkyltriazenes were metabolised at a substrate concentration of 500 $\mu g\ ml^{-1}$ by 9000g liver fractions from

Table 21 Protein content of microsomal preparations

Animal	Protein content ($\mu g\ mg^{-1}$ liver)
Female CBA/Lac mouse	21.07 ± 2.0
Female BK/Wistar rat	22.65 ± 1.6
Phenobarbitone induced female rat	23.75 ± 0.6
Male CBA/Lac mouse	20.80 ± 0.3

male CBA/Ca mice under closed bioassay conditions. The levels of metabolites produced are shown in figures 26 - 31; no more than 20% of the substrate was metabolised over the 2 hour incubation. All of the dialkyltriazenes were stable in control incubations containing liver preparations inactivated by heating.

D.2.4 Metabolism of monoalkyltriazenes and arylamines

Metabolism of three monomethyltriazenes (AcMMT;Ik, CyMMT;Ir, Idd) by 9000g fractions from male CBA/Ca mice was studied at a concentration of 4 $\mu g\ ml^{-1}$. The disappearance of substrate was measured by a reverse phase HPLC assay (section C.2.10.2) for up to 30 minutes and compared with the disappearance in control incubates containing liver inactivated by heating, no liver, or no cofactors. The results are shown in figures 32 - 34.

The metabolism of two arylamines (Xa, Xb) was investigated in the same way; these results are shown in figures 35 and 36.

Higher concentrations of triazenes were used to investigate comparisons between the stability and the rate of metabolism of monomethyl- and monoethyl- triazenes. Experimental (E) and control (C) incubations had the same composition except that, in controls, 9000g liver fractions inactivated by heating were used. The disappearance of AcMMT and AcMET (In) (100 $\mu g\ ml^{-1}$) is shown in figure 37, comparing control and experimental incubates in each case. Figure 38 shows the results of identical experiments using CyMMT and CyMET (It). Half-lives were calculated from the

Table 22 Half-lives (min) of monoalkyltriazenes at 37°C under different conditions

Conditions	Monoaklyltriazene			
	AcMMT	AcMET	CyMMT	CyMET
In control	33.4 ±	11.4 ±	59.6 ±	22.2 ±
incubate (control $t_{\frac{1}{2}}$)	4.5	1.7	4.7	2.1
In experimental	17.7 ±	5.6 ±	39.3 ±	16.7 ±
incubate (100 µg ml^{-1}) (metabolic $t_{\frac{1}{2}}$)	2.9	1.1	3.0	0.7
As above	-	-	31.8 ±	14.5 ±
(50 µg ml^{-1})			8.7	2.1
In Earl's buffer pH 7.4 (n=2) (chemical $t_{\frac{1}{2}}$)	66.6	15.1	99.0	18.3

data shown in these figures, and are presented in table 22. Also in table 22 are the half-lives in Earl's buffer pH 7.4 determined by HPLC.

Figures 39 and 40 show the disappearance of CyMMT and CyMET in control and experimental incubates at a substrate concentration of 50 μg ml^{-1}. Also shown is the appearance of 4-aminobenzonitrile (Xb).

In table 23, the amount of each of the monoalkyltriazenes decomposed in 30 minutes in experimental and control incubates is shown. From these values the theoretical arylamine concentrations was calculated assuming that the monoalkyltriazene had only been converted to the respective arylamine. By comparing control and experimental incubates for each substrate, an indication of the proportion of monoalkyltriazene either metabolised or chemically degraded in the experimental system may be obtained. Also in table 23, a comparison is made between the percentage of the theoretical arylamine concentration actually detected by HPLC and the percentage of each substrate decomposed which is chemically degraded in the experimental incubates.

D.2.5 Metabolism of 1-(4-acetylphenyl)-3-methyltriazene (AcMMT) (Ik) by TLX5 lymphoma ascites cells

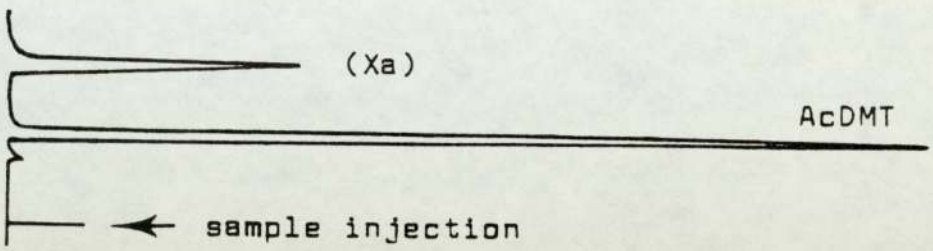
The disappearance of AcMMT was followed for up to 2 hours in the presence of triazene sensitive (TLX5) or triazene resistant (TLX5R) cell lines, and assayed by reverse phase HPLC. In the experimental incubations the triazene concentration (5 μg in each 1.5 ml incubate - 18.8 μM) was

Table 23 Amount of monoalkyltriazene decomposed and theoretical arylamine concentrations if conversion only to the respective arylamine; and comparison of the percentage of theoretical arylamine concentration detected with percentage of substrate decomposed in experimental incubates chemically degraded

Substrate	Amount monoaklyltriazene decomposed in experimental incubates (mg) (control in brackets)	Theoretical arylamine concentration if conversion of monoaklyltriazine only to arylamine ($\mu g\ ml^{-1}$) *	% of theoretical concentration of arylamine detected by HPLC *	% of substrate decomposed in experimental incubates chemically degraded
AcMMT ($100\ \mu g\ ml^{-1}$)	180.75 (115.75) $\pm$20.3 ($\pm$ 12.5)	(Xa) 55.1 (35.3)	(Xa) 70.4 (91.8)	64.0
AcMET ($100\ \mu g\ ml^{-1}$)	243.68 (205.25) $\pm$ 5.3 ($\pm$ 7.5)	68.9 (58.0)	92.7(102.2)	84.2
CyMMT ($50\ \mu g\ ml^{-1}$)	60.88 (20.25) $\pm$12.0 ($\pm$10.9)	(Xb) 18.0 (6.0)	(Xb) 58.3(105.0)	33.2
CyMET ($50\ \mu g\ ml^{-1}$)	95.25 (68.75) $\pm$6.1 ($\pm$ 5.3)	25.8 (18.6)	79.7 (94.6)	72.2

* First value that in experimental incubate, second value (in brackets) in control incubate

too low to elicit toxicity to the cells as determined by the trypan blue dye exclusion test (section C.3.3) Control incubates contained cells inactivated by sonication or heating. Both control incubations gave identical disappearance rates, results are shown in figure 41 for cells disrupted by sonication. There was no difference in the percentage of AcMMT which disappeared in 2 hours in control and experimental incubates using either sensitive or resistant cell lines.



Compound	Retention time (min)
AcDMT	4.75
(Xa)	8.50

Figure 11 HPLC separation of 1-(4-acetylphenyl)-3,3-dimethyltriazene (AcDMT) and 4-aminoacetophenone (Xa)

For conditions, see section C.2.10.1

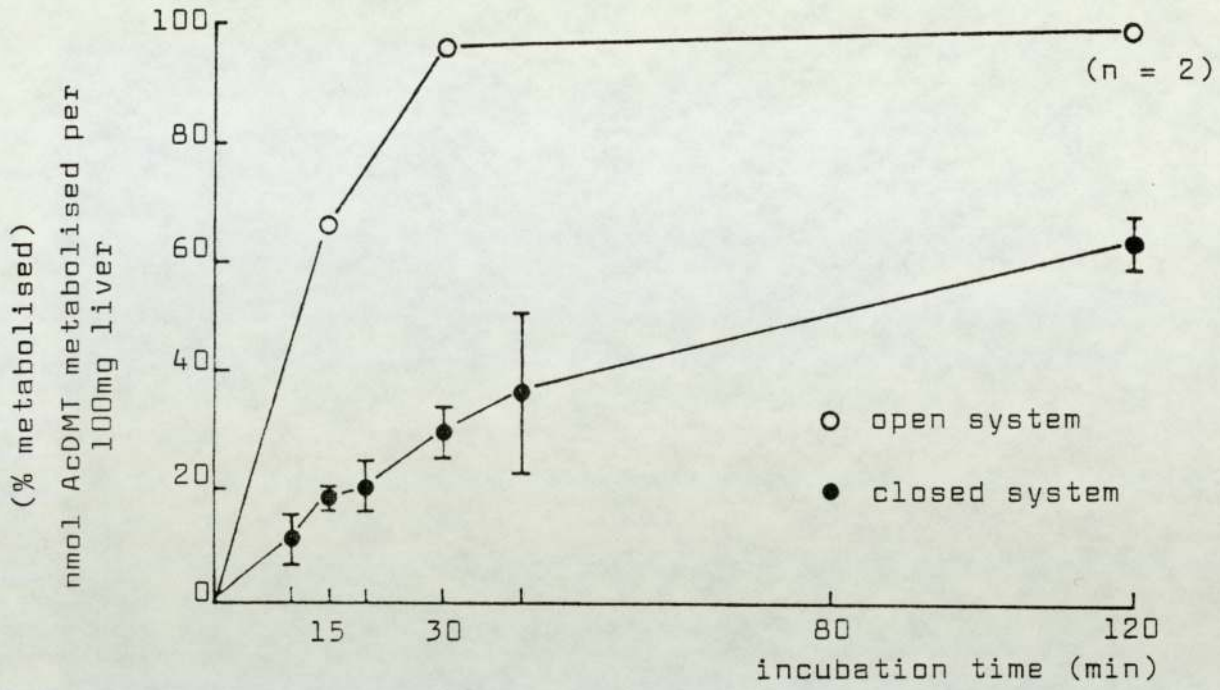


Figure 12 Metabolism of AcDMT (50 μM) by the 9000g liver fraction from male CBA/Lac mice.

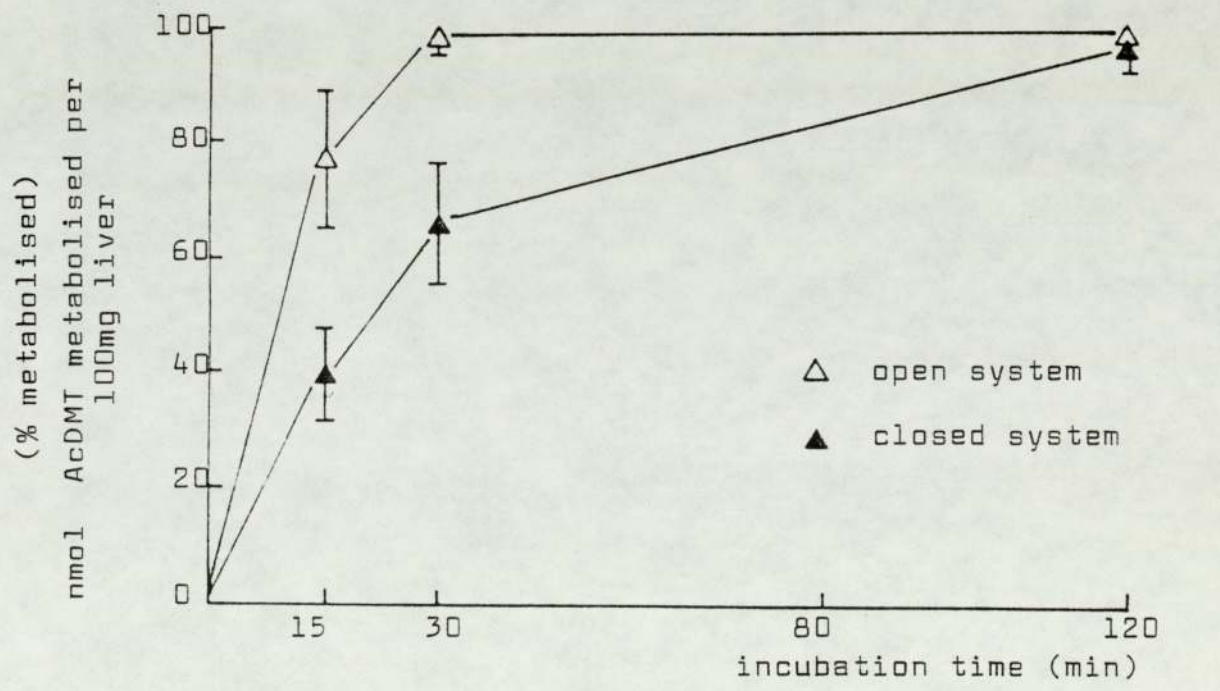


Figure 13 Metabolism of AcDMT (50 μM) by the 9000g liver fraction from female rats.

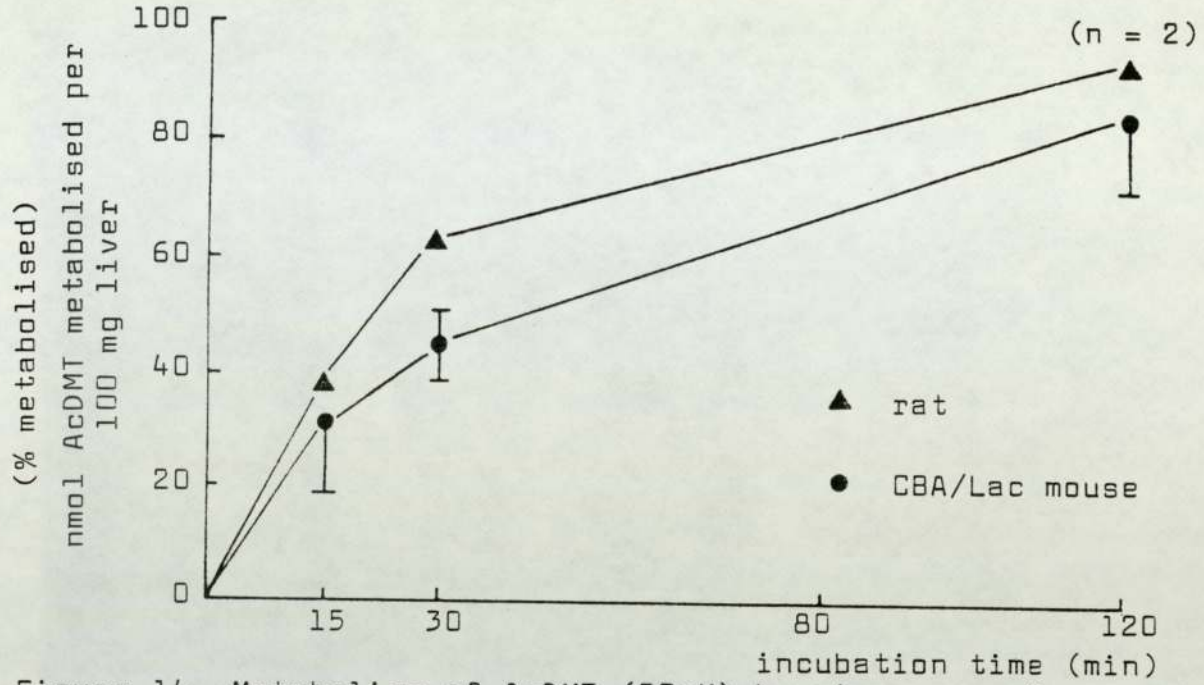


Figure 14 Metabolism of AcDMT (50μM) by microsomes from female animals.

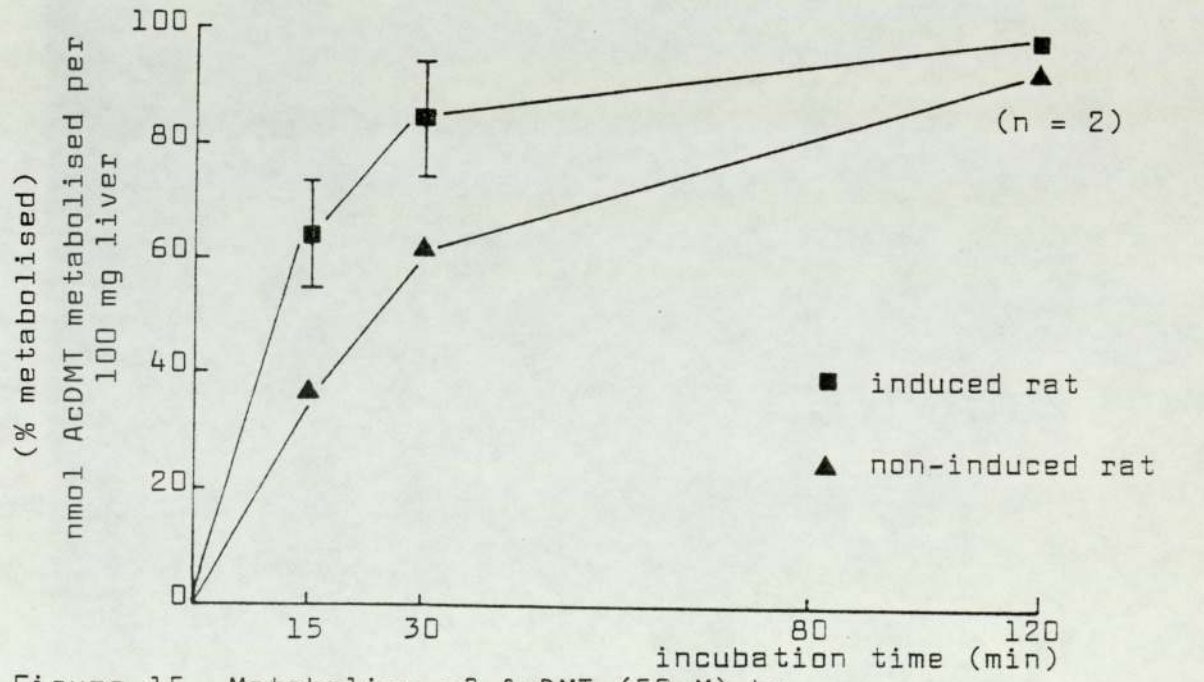


Figure 15 Metabolism of AcDMT (50μM) by microsomes from induced and non-induced female rats.

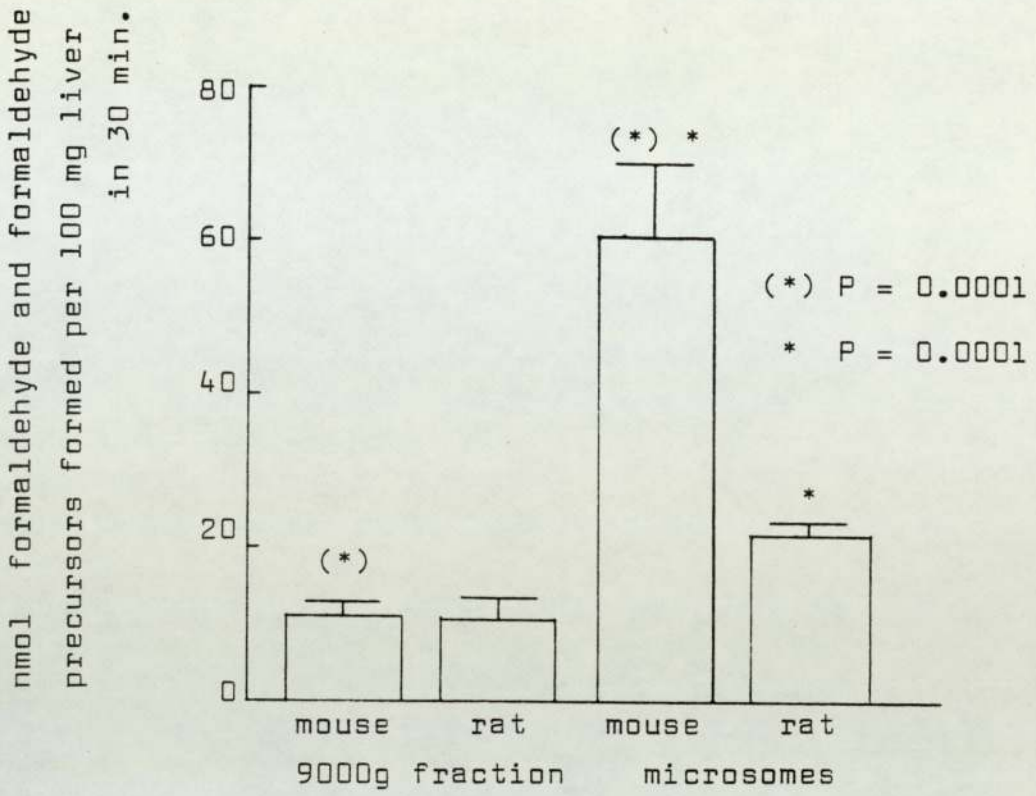


Figure 16 Metabolism of aminopyrine (4mM) by liver preparations from female CBA/Lac mice and female rats (open system)

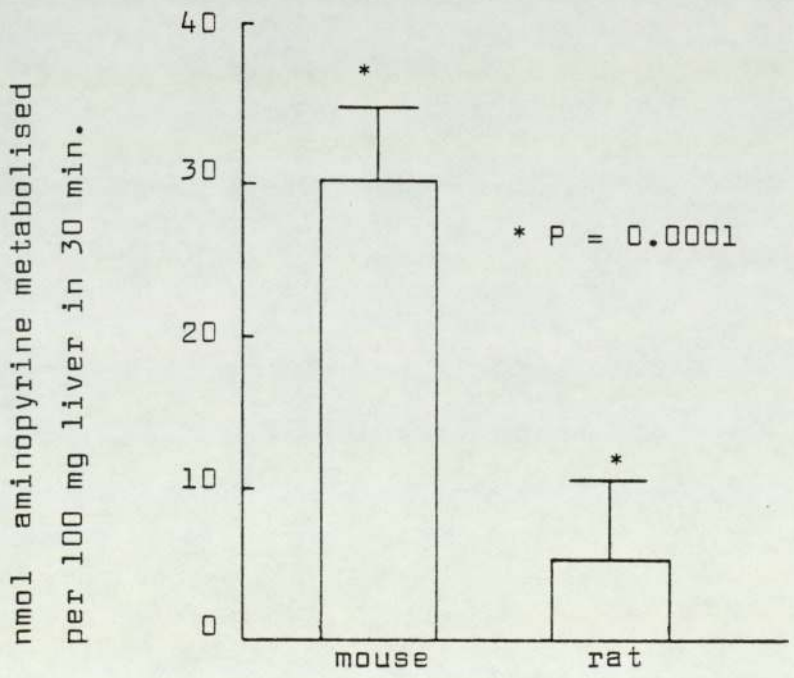


Figure 17 Metabolism of aminopyrine (50 μM) by microsomes from female CBA/Lac mice and female rats (open system)

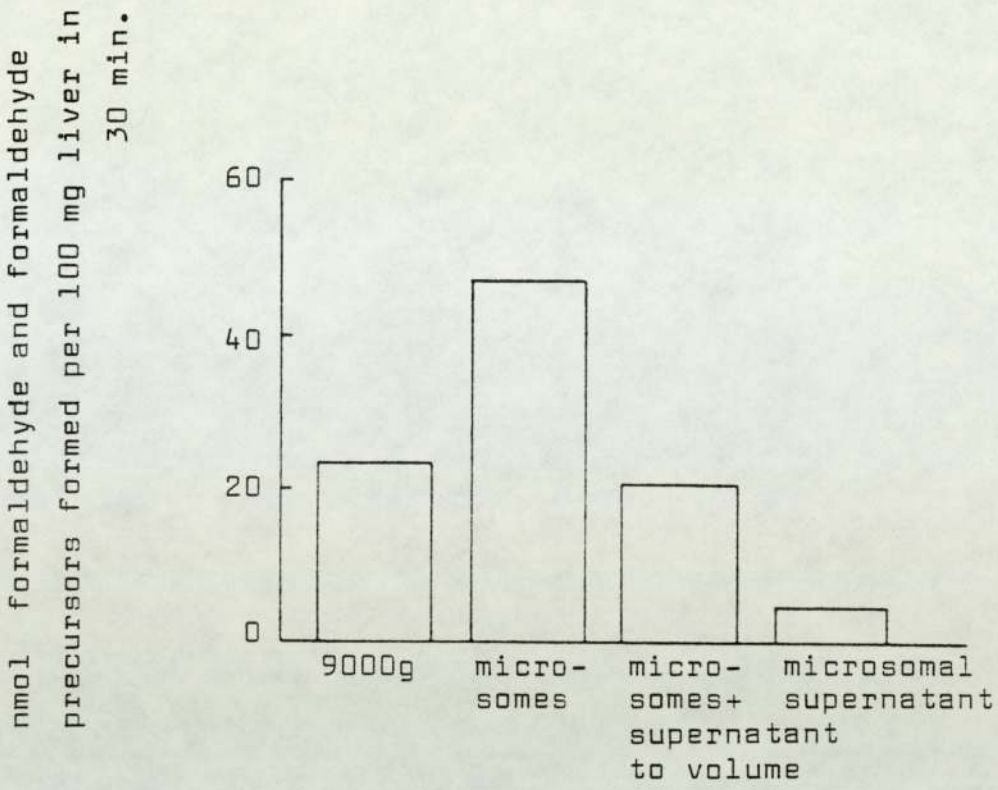


Figure 18 Metabolism of aminopyrine (4mM) by liver preparations from male CBA/Lac mice

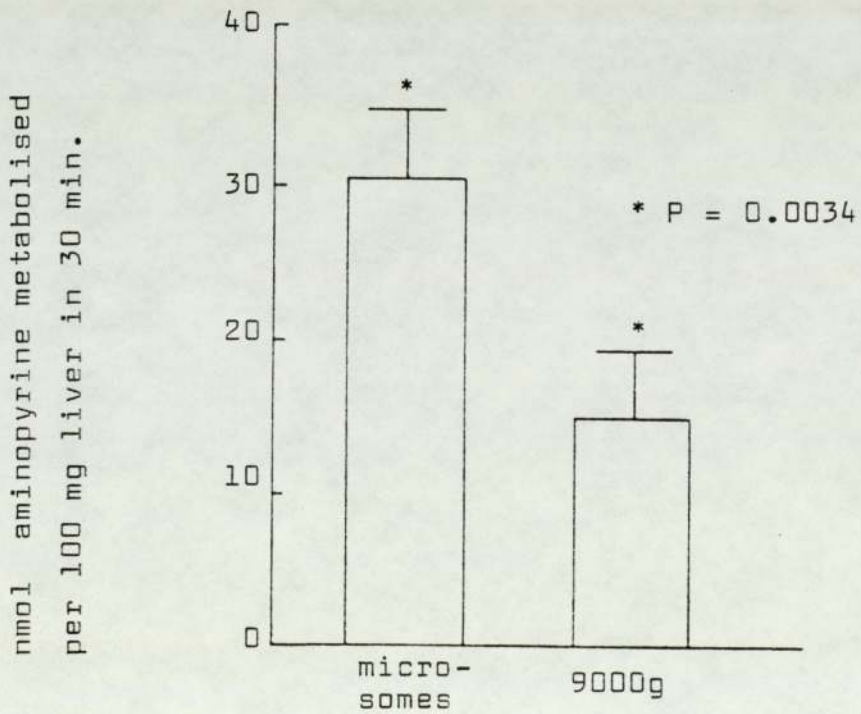


Figure 19 Metabolism of aminopyrine (50 μM) by liver preparations from female CBA/Lac mice.

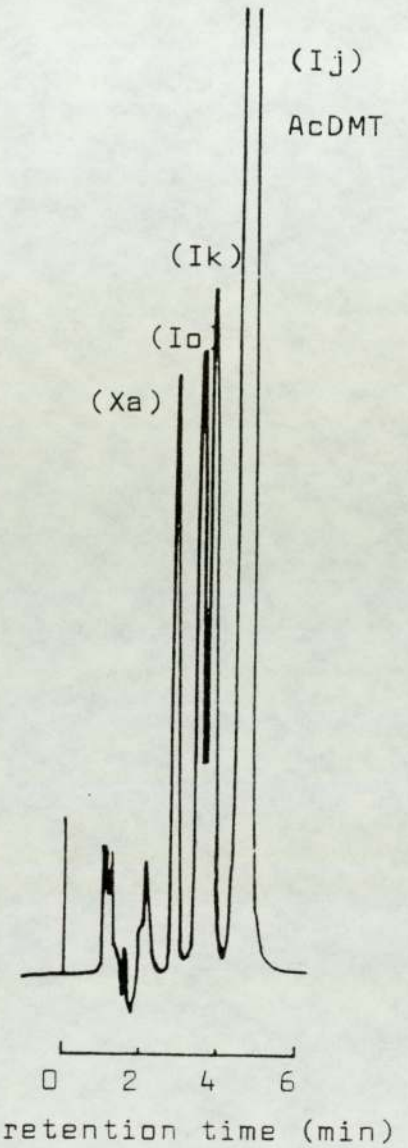


Figure 20 HPLC separation of 1-(4-acetylphenyl)-3,3-dimethyltriazene (AcDMT) and its potential metabolites

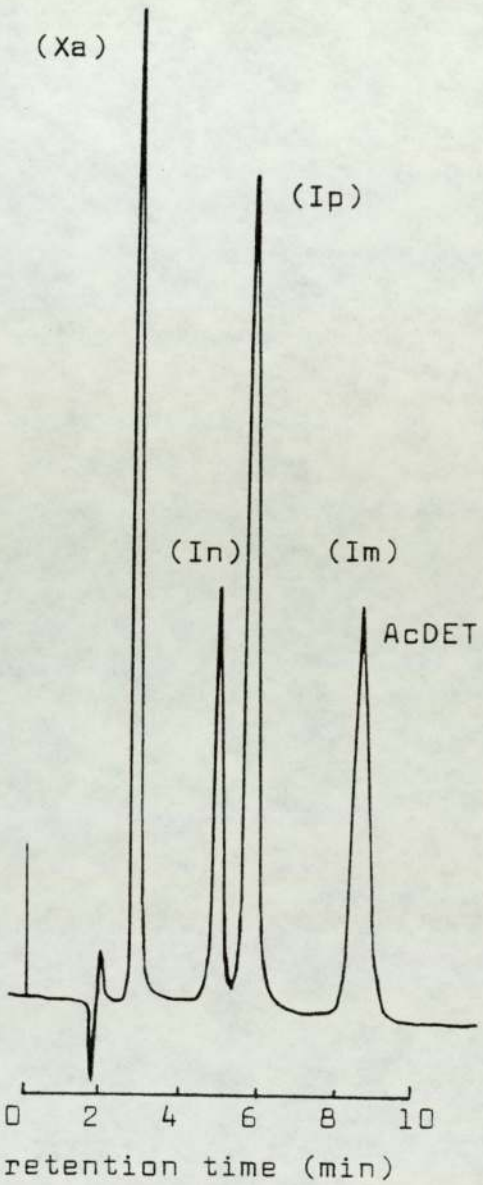


Figure 21 HPLC separation of 1-(4-acetylphenyl)-3,3-diethyltriazene (AcDET) and its potential metabolites

(Xa) 4-aminoacetophenone, (Ik) AcMMT, (In) AcMET,
(Io) 1-[4-(1-hydroxyethyl)phenyl]-3,3-dimethyltriazene,
(Ip) 1-[4-(1-hydroxyethyl)phenyl]-3,3-diethyltriazene

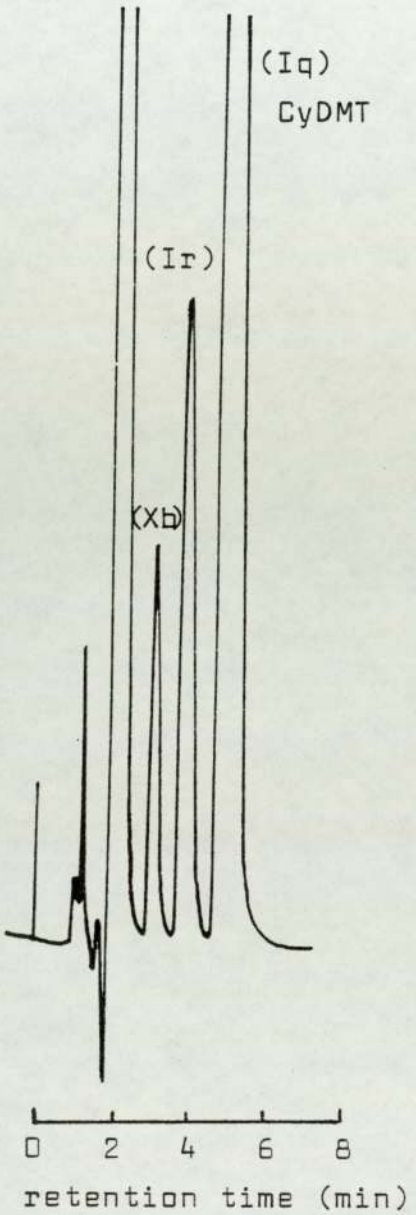


Figure 22 HPLC separation of 1-(4-cyanophenyl)-3,3-dimethyltriazene (CyDMT) and its potential metabolites

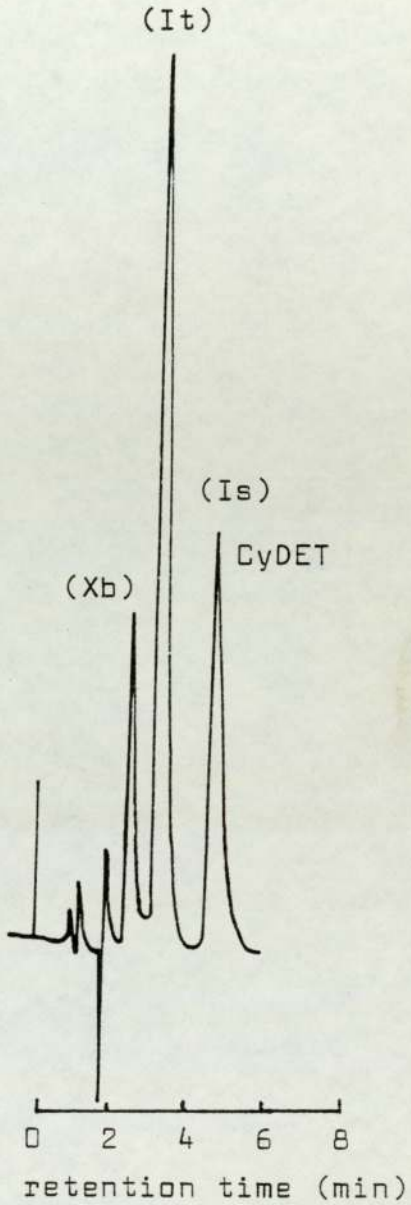


Figure 23 HPLC separation of 1-(4-cyanophenyl)-3,3-diethyltriazene (CyDET) and its potential metabolites

(Xb) 4-aminobenzonitrile, (Ir) CyMMT, (It) CyMET

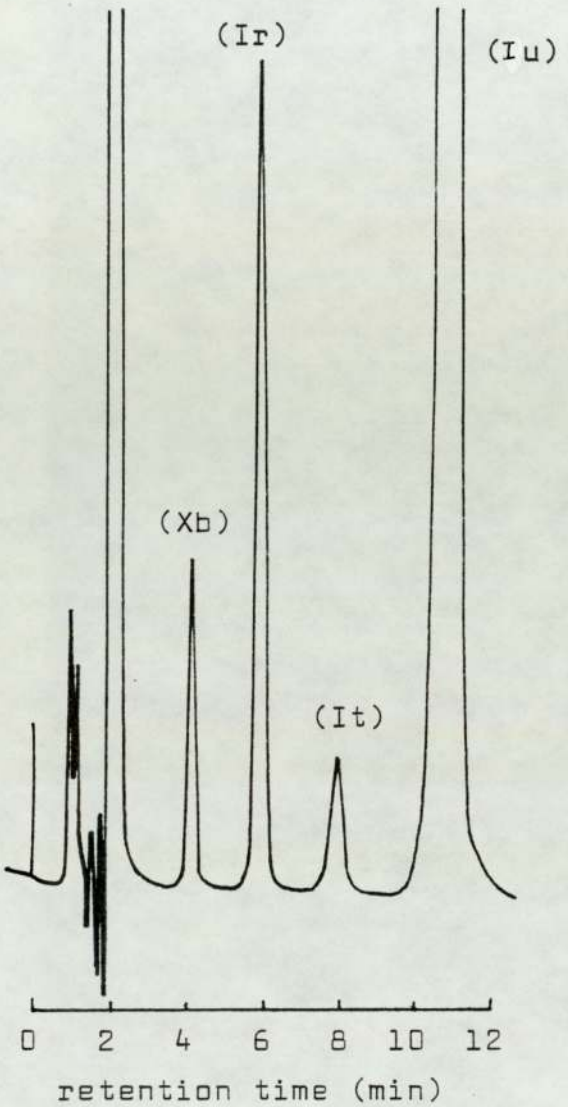


Figure 24 HPLC separation of 1-(4-cyanophenyl)-3-methyl-3-ethyltriazene and its potential metabolites

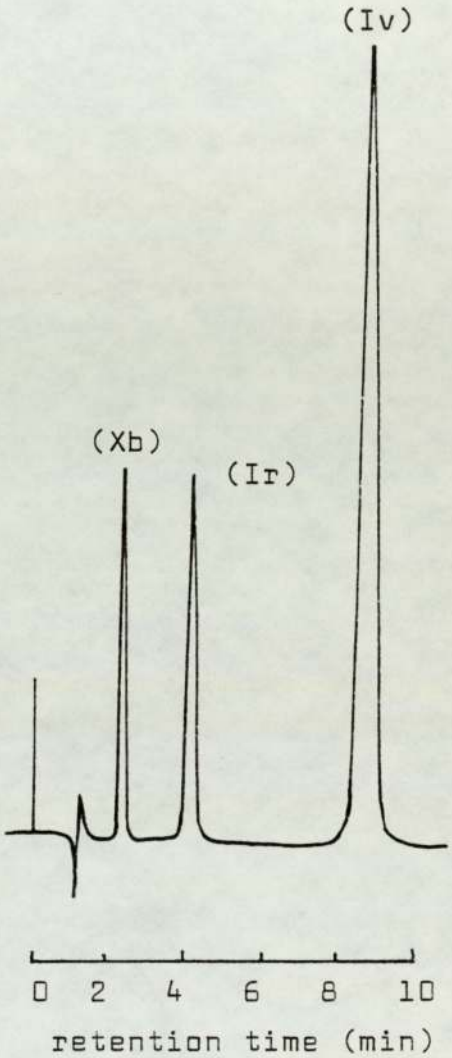


Figure 25 HPLC separation of 1-(4-cyanophenyl)-3-methyl-3-t-butyltriazene and its potential metabolites

(Xb) 4-aminobenzonitrile, (Ir) CyMMT, (It) CyMET

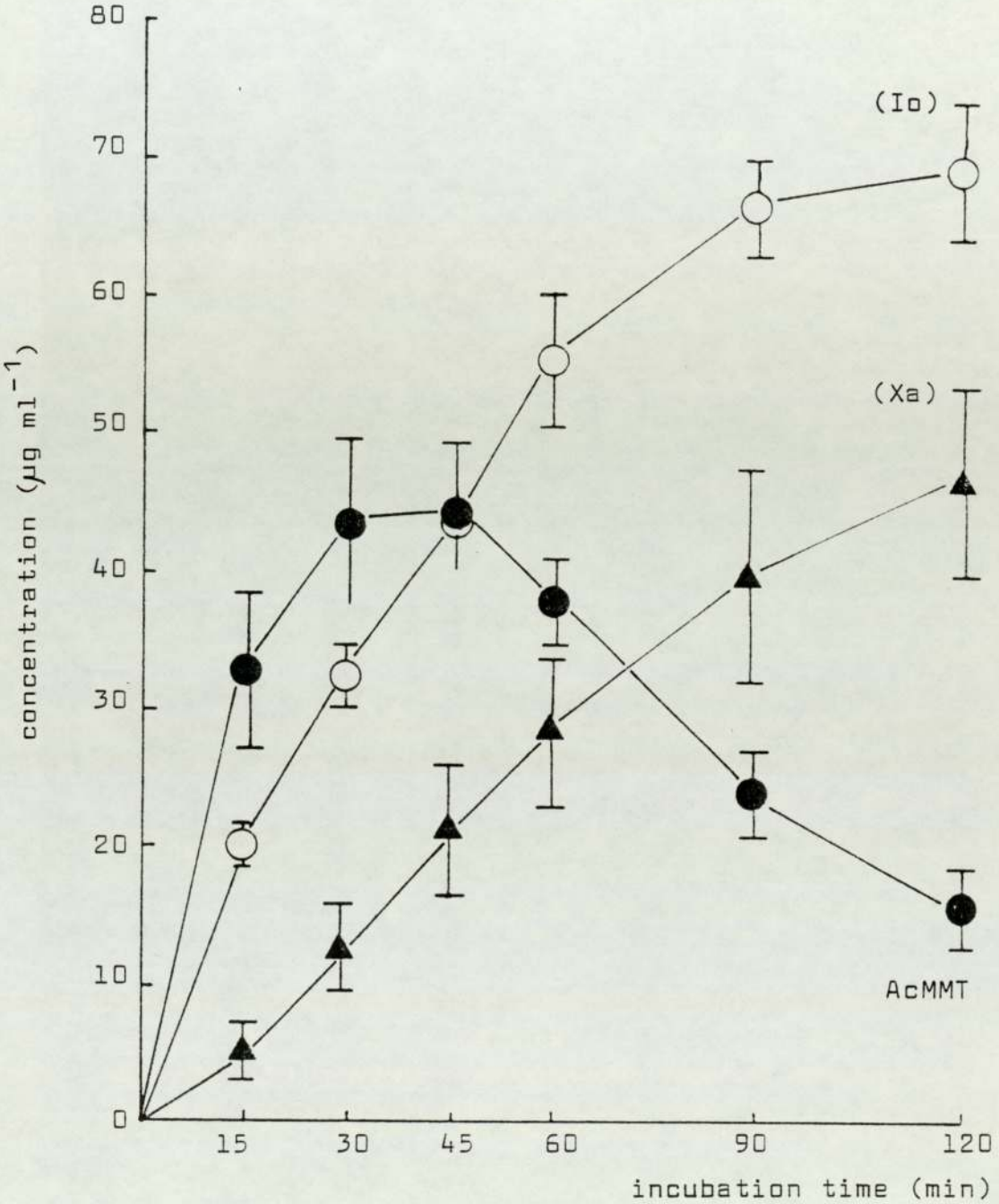


Figure 26 Products yielded on metabolism of AcDMT (500 μg ml^{-1}) by mouse 9000g liver fraction.

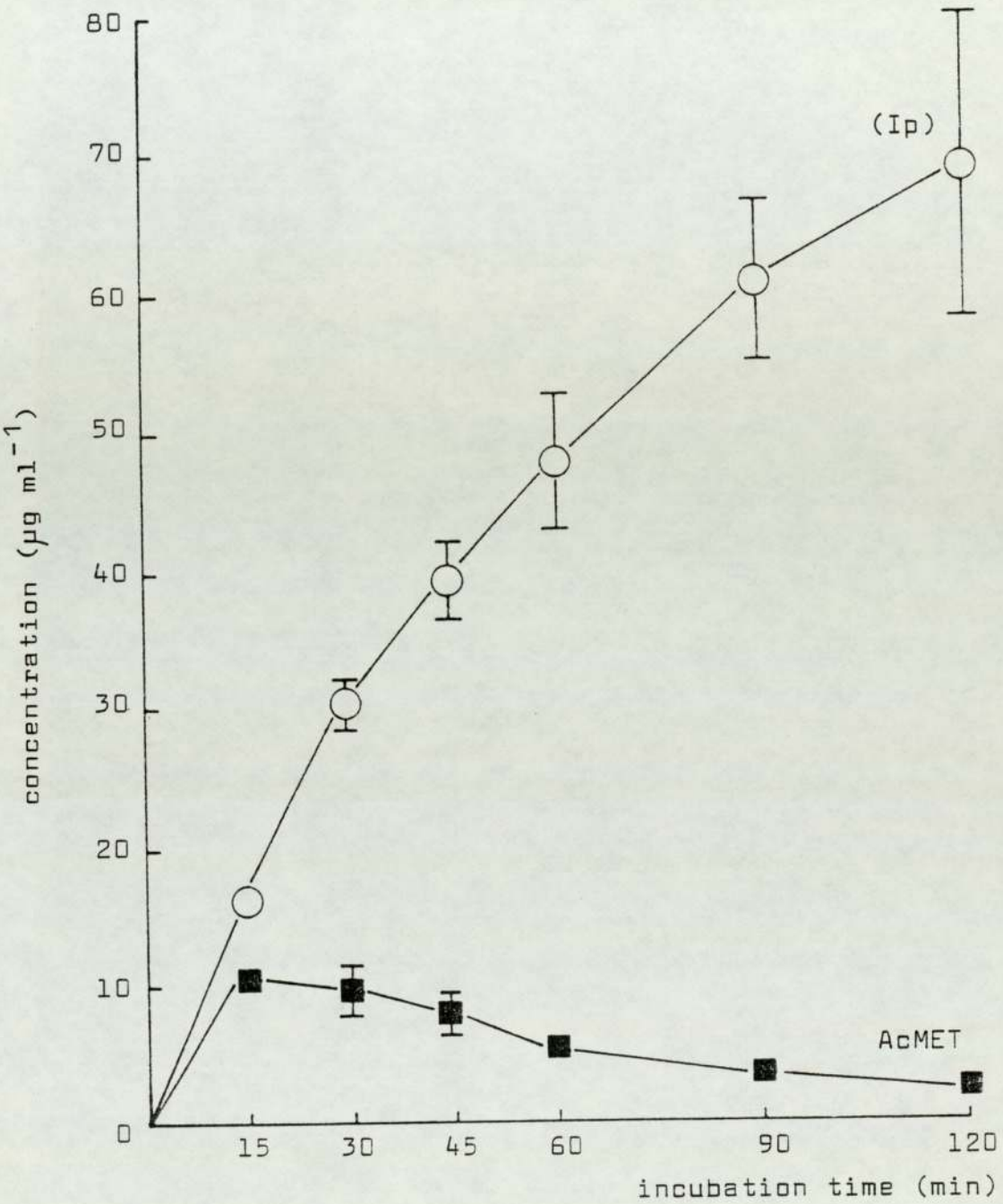


Figure 27 Products yielded on metabolism of AcDET (500 μg ml^{-1}) by mouse 9000g liver fraction

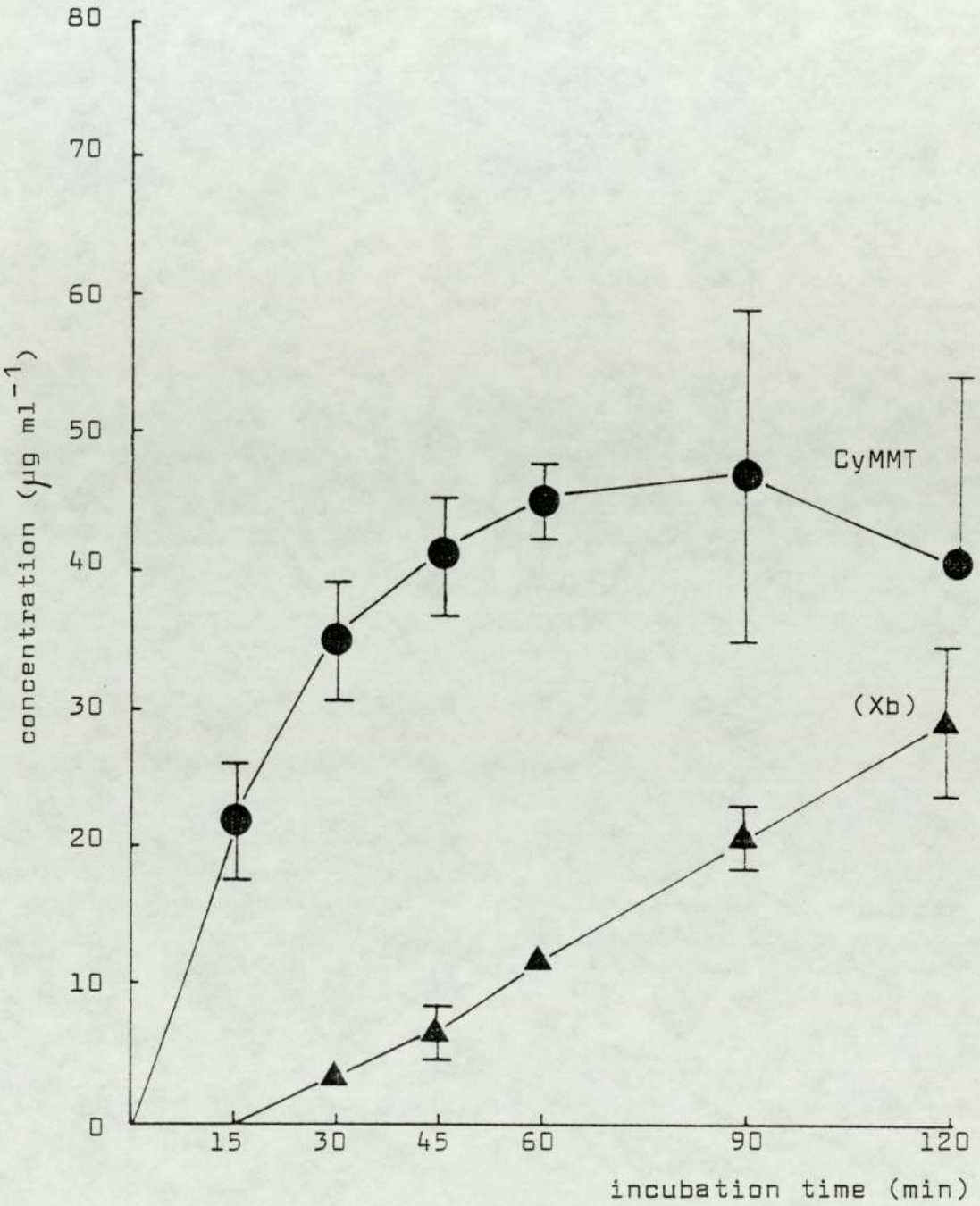


Figure 28 Products yielded on metabolism of CyDMT (500 µg ml^{-1}) by mouse 9000g liver fraction

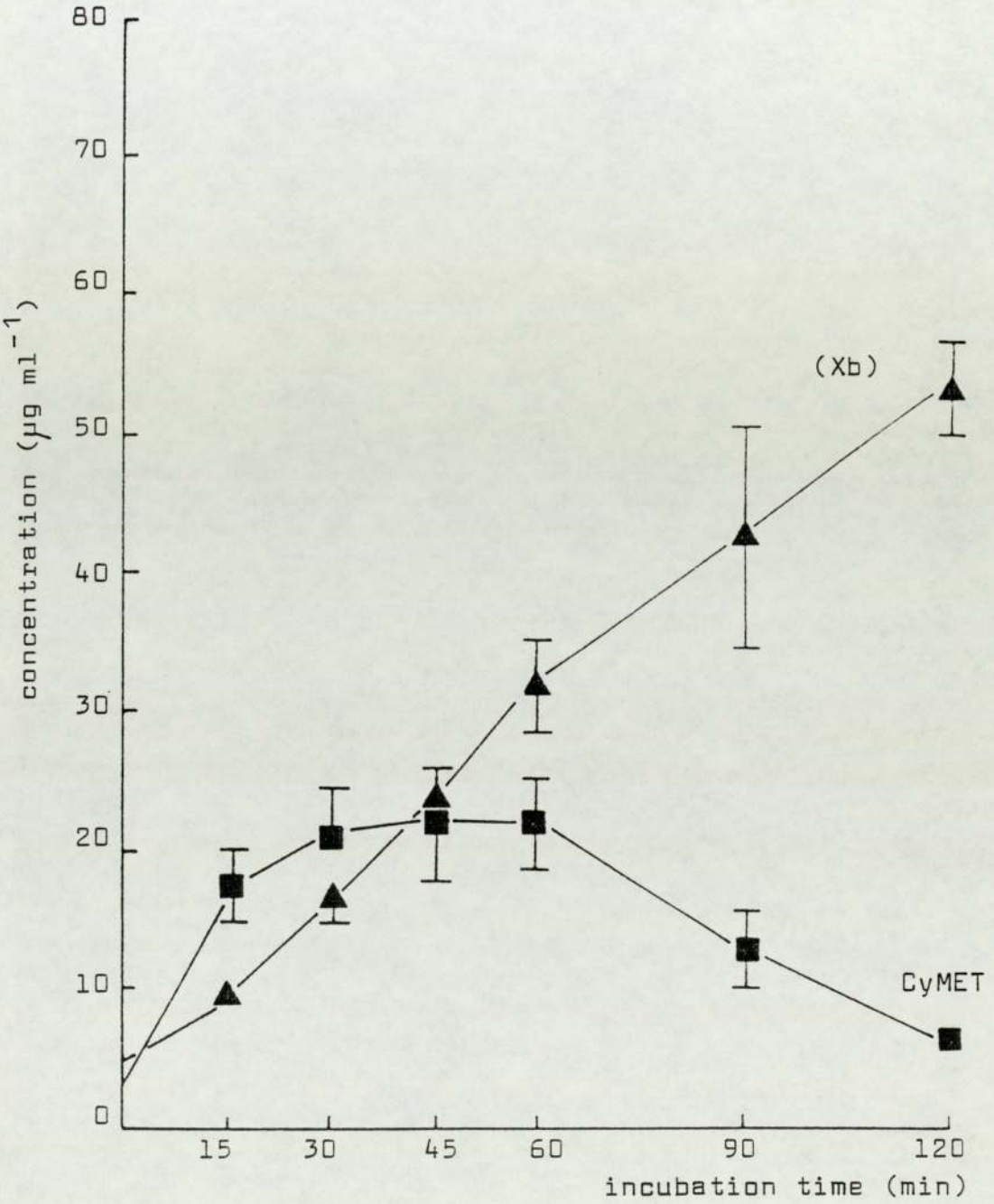


Figure 29 Products yielded on metabolism of CyDET (500 µg ml^{-1}) by mouse 9000g liver fraction

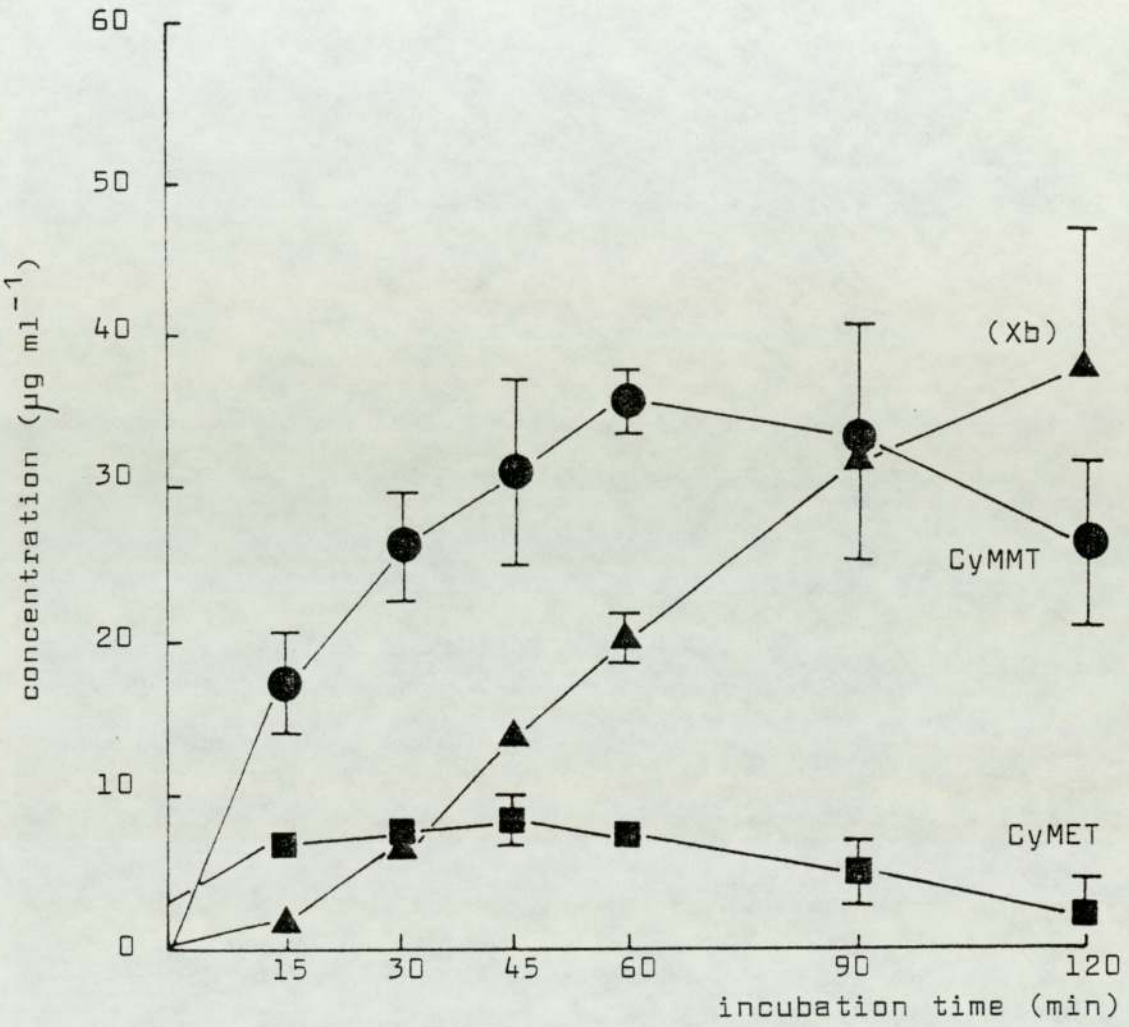


Figure 30 Products yielded on metabolism of 1-(4-cyanophenyl)-3-methyl-3-ethyltriazene (Iu) (500 µg ml^{-1}) by mouse 9000g liver fraction

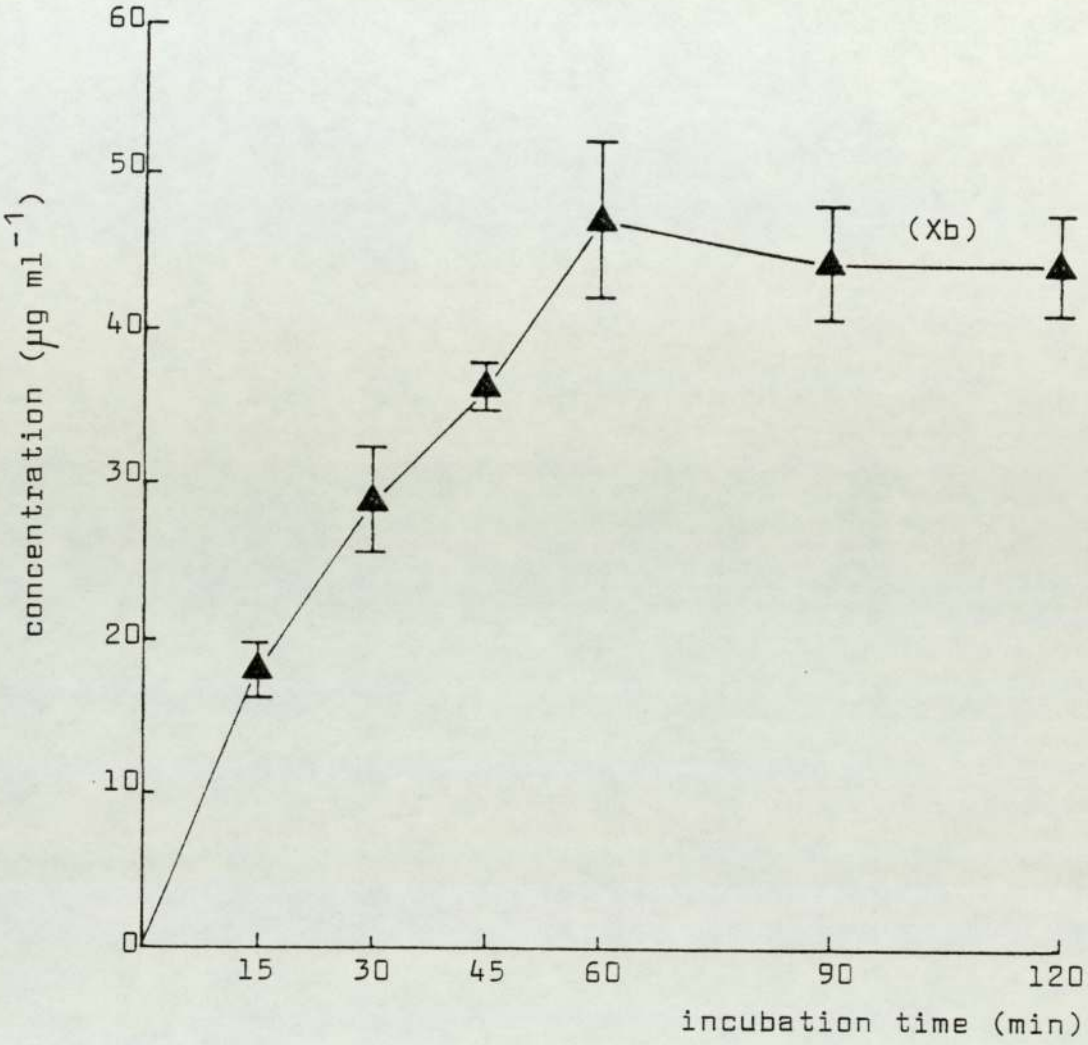


Figure 31 Product yielded on metabolism of 1-(4-cyanophenyl)-3-methyl-3-tert-butyltriazene (Iv) (500 μg ml^{-1}) by mouse 9000g liver fraction.

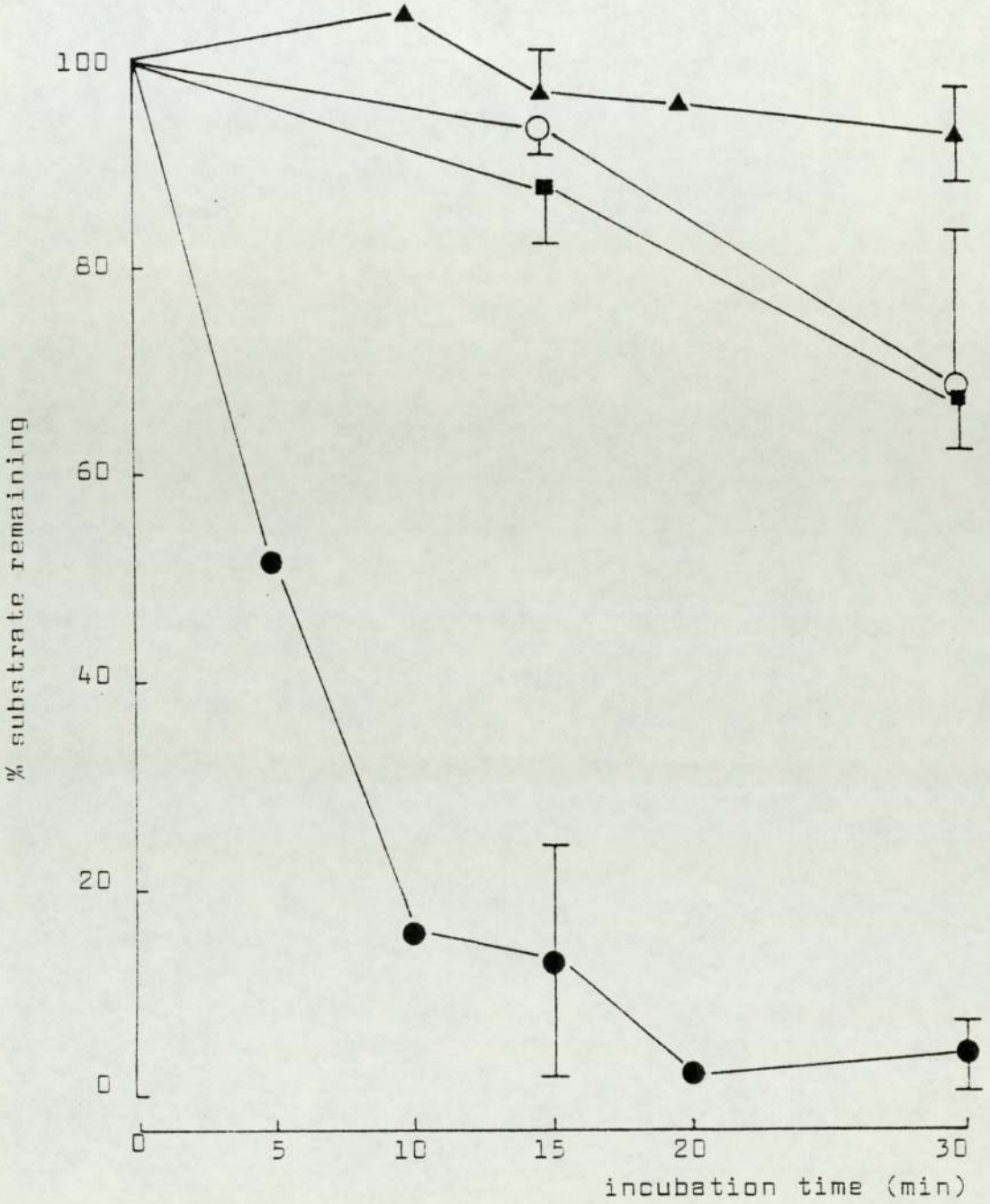


Figure 32 Disappearance of AcMMT (4 μg ml^{-1}) under different incubation conditions.

▲ mouse 9000g liver fraction only

○ buffer + cofactors

■ heat inactivated liver + cofactors

● 9000g liver fraction + cofactors

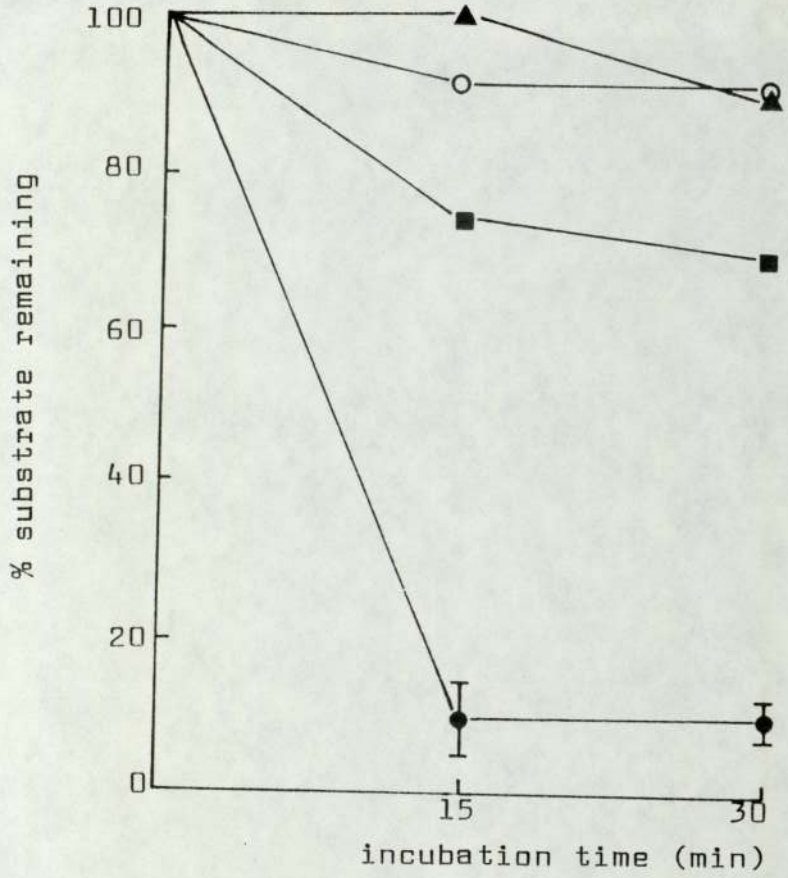


Figure 33 Disappearance of CyMMT (4 μg ml^{-1}) under different incubation conditions.

▲ mouse 9000g liver fraction only

■ heat inactivated liver + cofactors

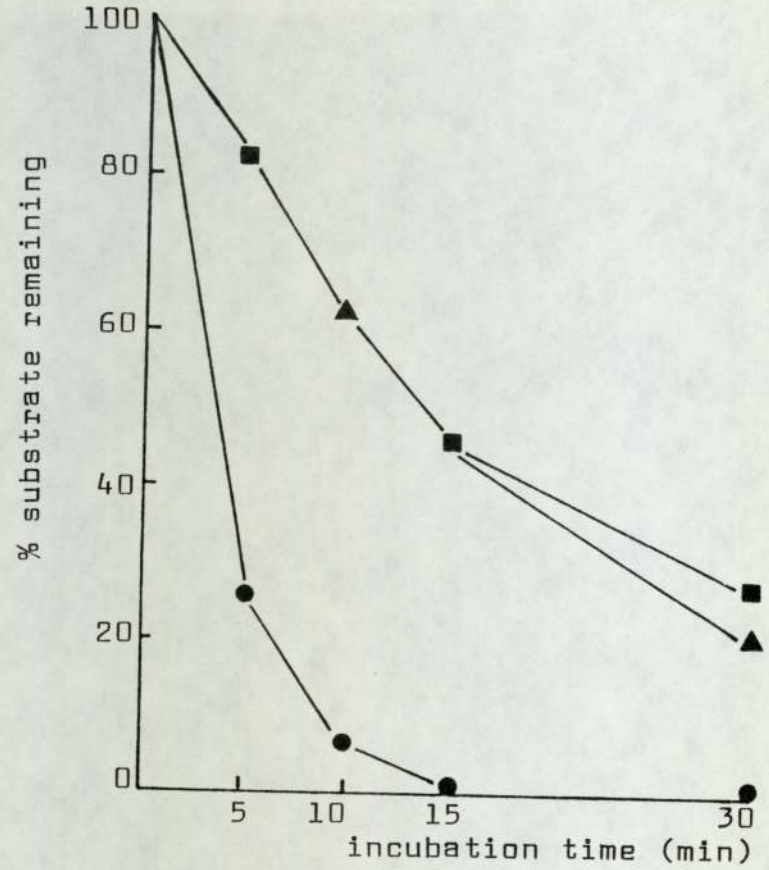


Figure 34 Disappearance of 1-(4-nitrophenyl)-3-methyltriazene (Idd)(4 μg ml^{-1}) under different incubation conditions.

○ buffer + cofactors

● 9000g liver fraction + cofactors

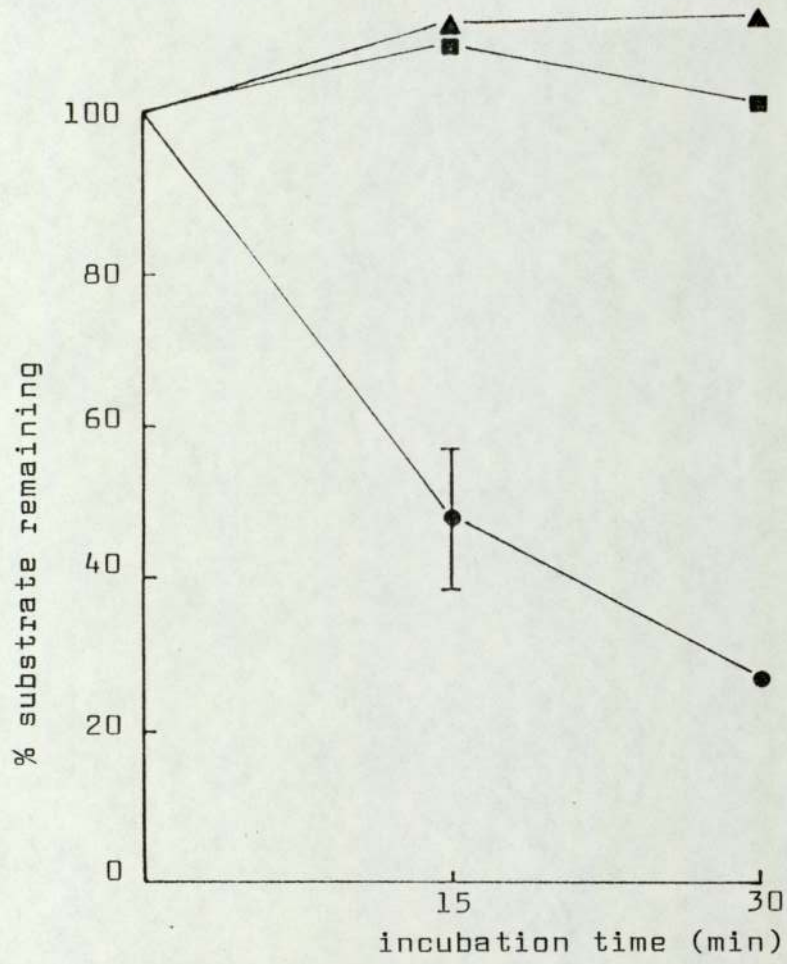


Figure 35 Disappearance of 4-aminoacetophenone ($4\ \mu g\ ml^{-1}$) under different incubation conditions

▲ mouse 9000g liver fraction only

■ heat inactivated liver + cofactors

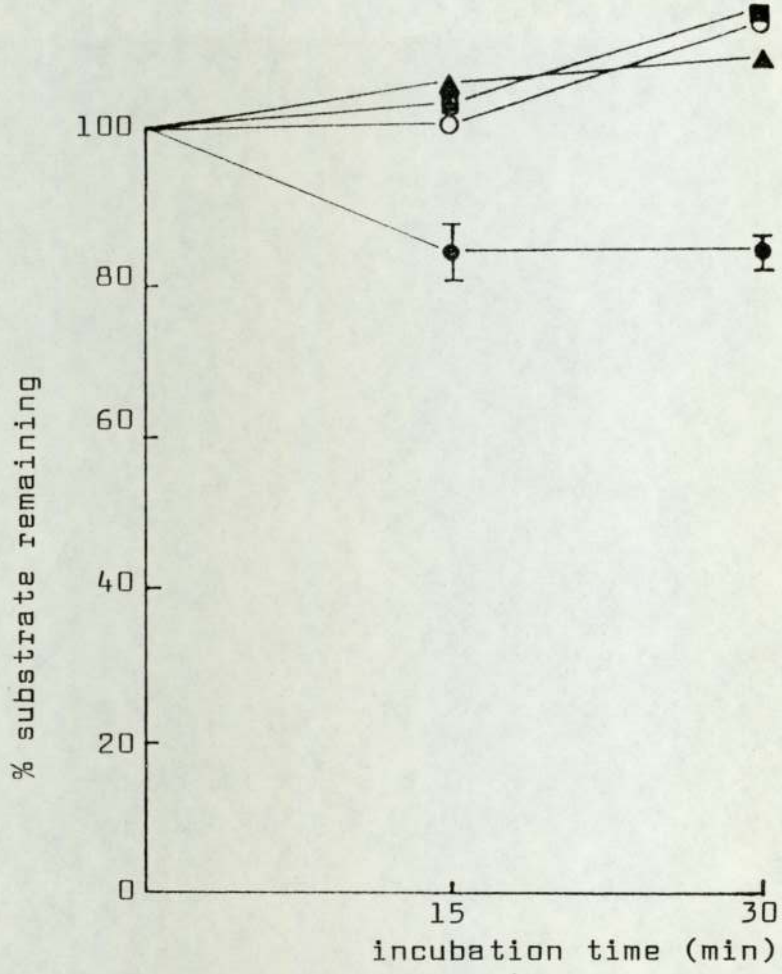


Figure 36 Disappearance of 4-aminobenzonitrile ($4\ \mu g\ ml^{-1}$) under different incubation conditions.

○ buffer + cofactors

● 9000g liver fraction + cofactors

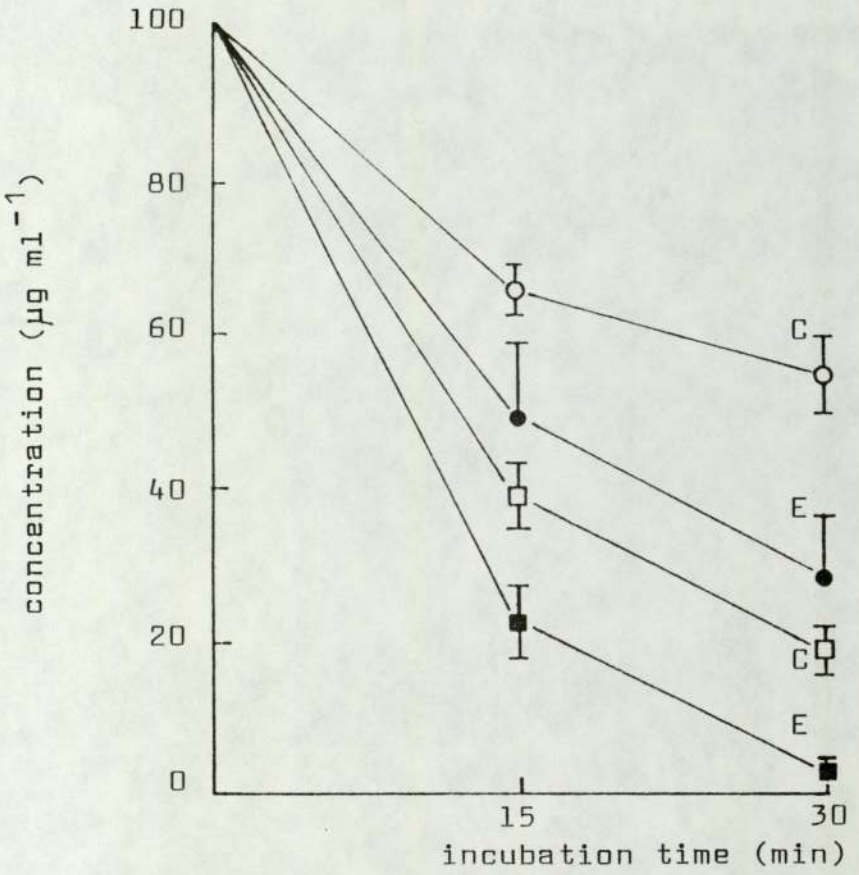


Figure 37 Disappearance of AcMMT (○ ●) and AcMET (□ ■)(100 μg ml^{-1}) in experimental (E) and control (C) incubates.

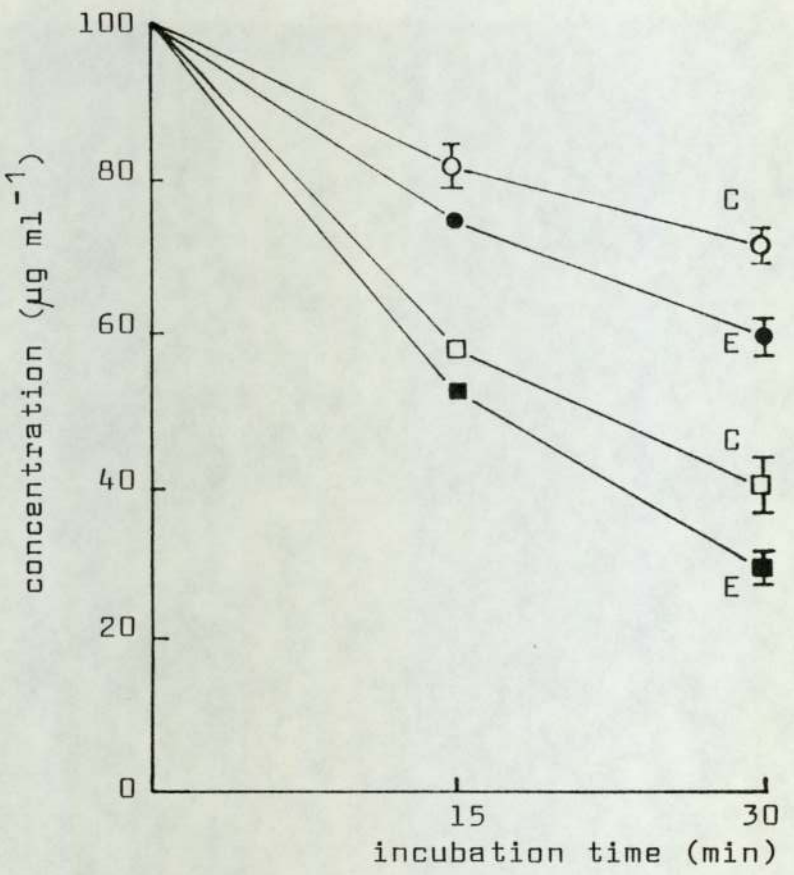


Figure 38 Disappearance of CyMMT (○ ●) and CyMET (□ ■)(100 μg ml^{-1}) in experimental (E) and control (C) incubates.

Experimental incubates contain mouse liver 9000g fraction, and control incubates the same liver preparation inactivated by heating.

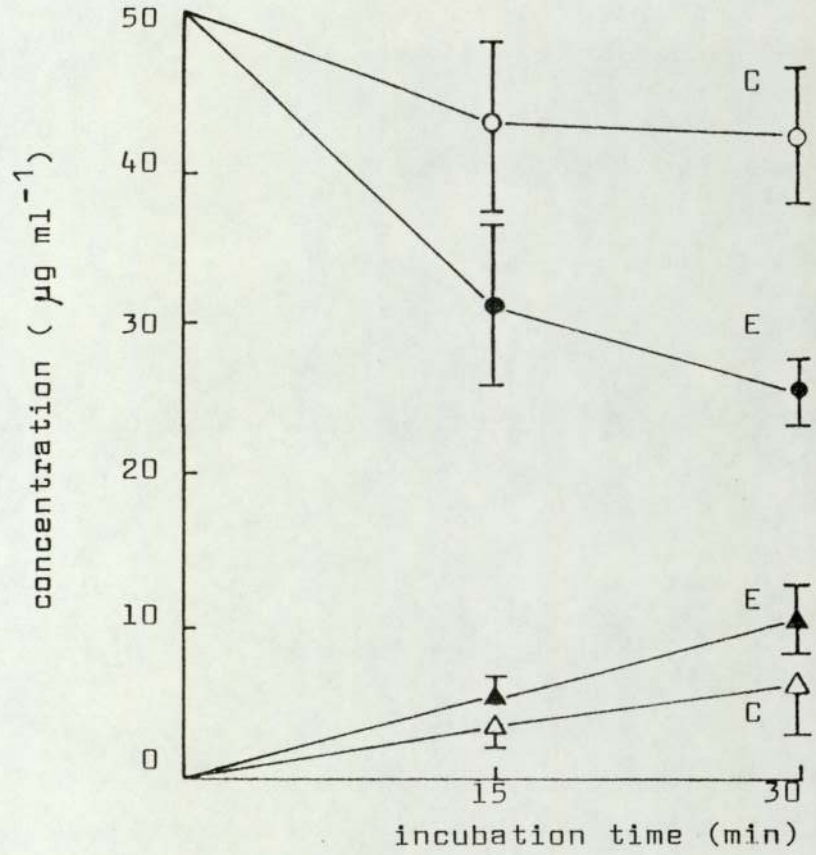


Figure 39 Disappearance of CyMMT (O ●) (50 µg ml^{-1}) and appearance of arylamine (Xb) (△▲) in experimental (E) and control (C) incubates.

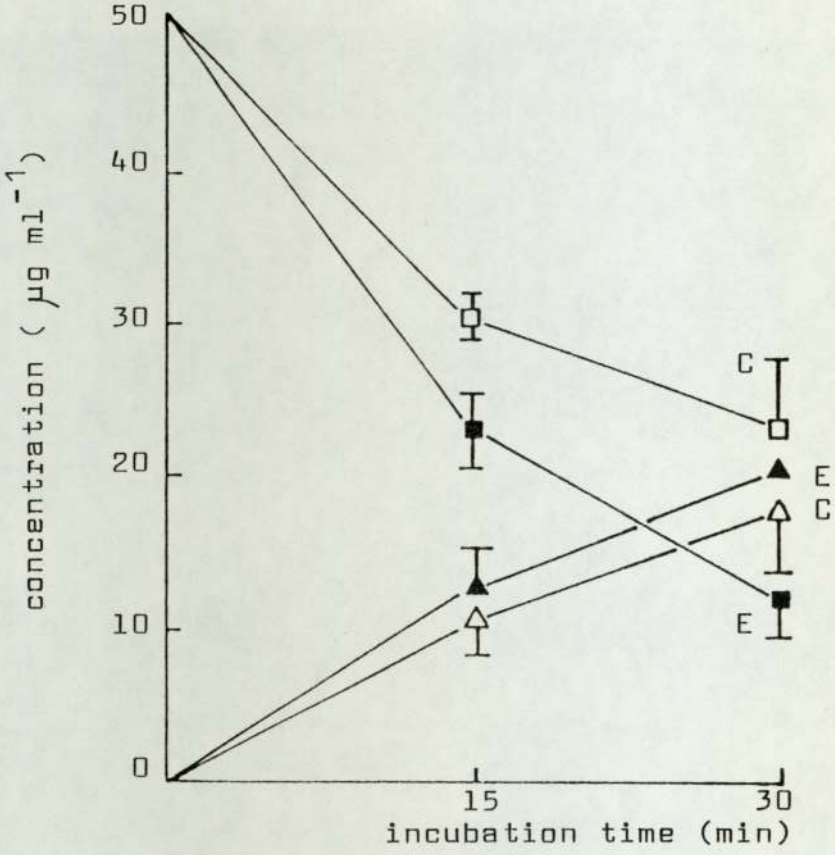


Figure 40 Disappearance of CyMET (□ ■) (50 µg ml^{-1}) and appearance of arylamine (Xb)(△▲) in experimental (E) and control (C) incubates.

Experimental incubates contain mouse liver 9000g fraction, and control incubates the same liver preparation inactivated by heating.

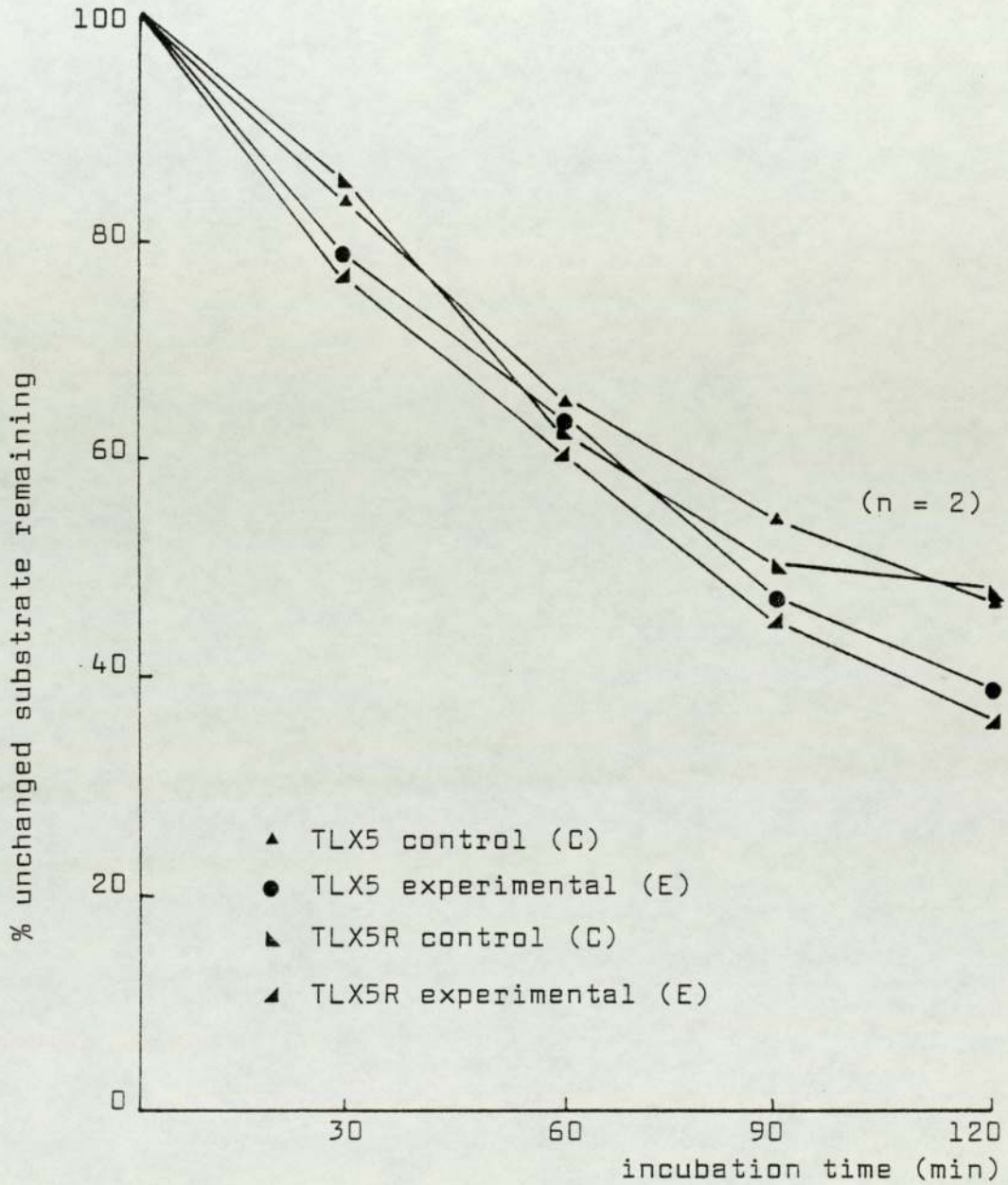


Figure 41 Disappearance of AcMMT (18.8 μM) in the presence of intact TLX5 or TLX5R cells (E) or cells disrupted by sonication (C)

D.3 Toxicity studies

D.3.1 Toxicity of 1-(4-acetylphenyl)-3,3-dimethyltriazene (AcDMT) (Ij) and 1-(4-acetylphenyl)-3-methyltriazene (AcMMT) (Ik) to mouse hepatocytes

The toxicity of AcDMT and AcMMT to hepatocytes was measured as a decrease in viability of the cells as determined by the trypan blue dye exclusion test (section C.3.1.3), when compared with control untreated hepatocytes. After one hour incubation with the triazenes at concentrations 0-1.5mM AcDMT and 0-2.5mM AcMMT, very little toxicity was seen except at the highest concentration of AcDMT employed (31% increase in trypan blue staining in experimental compared with control incubations). The results obtained after 2 hours are shown in figure 42. There was a significant difference between the toxicities of AcDMT and AcMMT at concentrations of 1.0mM ($P = 0.0016$) and 1.5mM ($P = 0.0070$).

The decrease in viability of the hepatocytes in response to the same concentration of triazene varied between cell preparations, causing large standard deviations. For example, at the 2 hour time point, the percentage increase in staining with 1.0mM AcDMT was 77.8 ± 28.8, and with 1.0mM AcMMT 16.6 ± 14.5. Error bars have therefore been omitted from figure 42 to aid clarity of presentation.

D.3.2 Toxicity of 1-(4-acetylphenyl)-3,3-dimethyltriazene (AcDMT) (Ij) and 1-(4-acetylphenyl)-3-methyltriazene (AcMMT) (Ik) to TLX5 lymphoma ascites cells

The toxicity of the triazenes was assayed by the decrease in viability of the TLX5 cells as determined by

trypan blue staining in comparison with control cells. Both sensitive (TLX5) and triazene resistant (TLX5R) cell lines were studied. The tumour cells were incubated with AcMMT at concentrations 0 - 1mg ml^{-1} (5.65mM) for two hours. The results are shown in figure 43. AcMMT was found to be more cytotoxic to the sensitive than to the resistant TLX5 line. The difference was significant at concentrations of 250 μg ml^{-1} ($P = 0.0015$) and 500 μg ml^{-1} ($P = 0.0022$). The dimethyltriazene, AcDMT, was non-toxic to both cell lines after incubation for 2 hours at a concentration of 1 mg ml^{-1} (5.24mM).

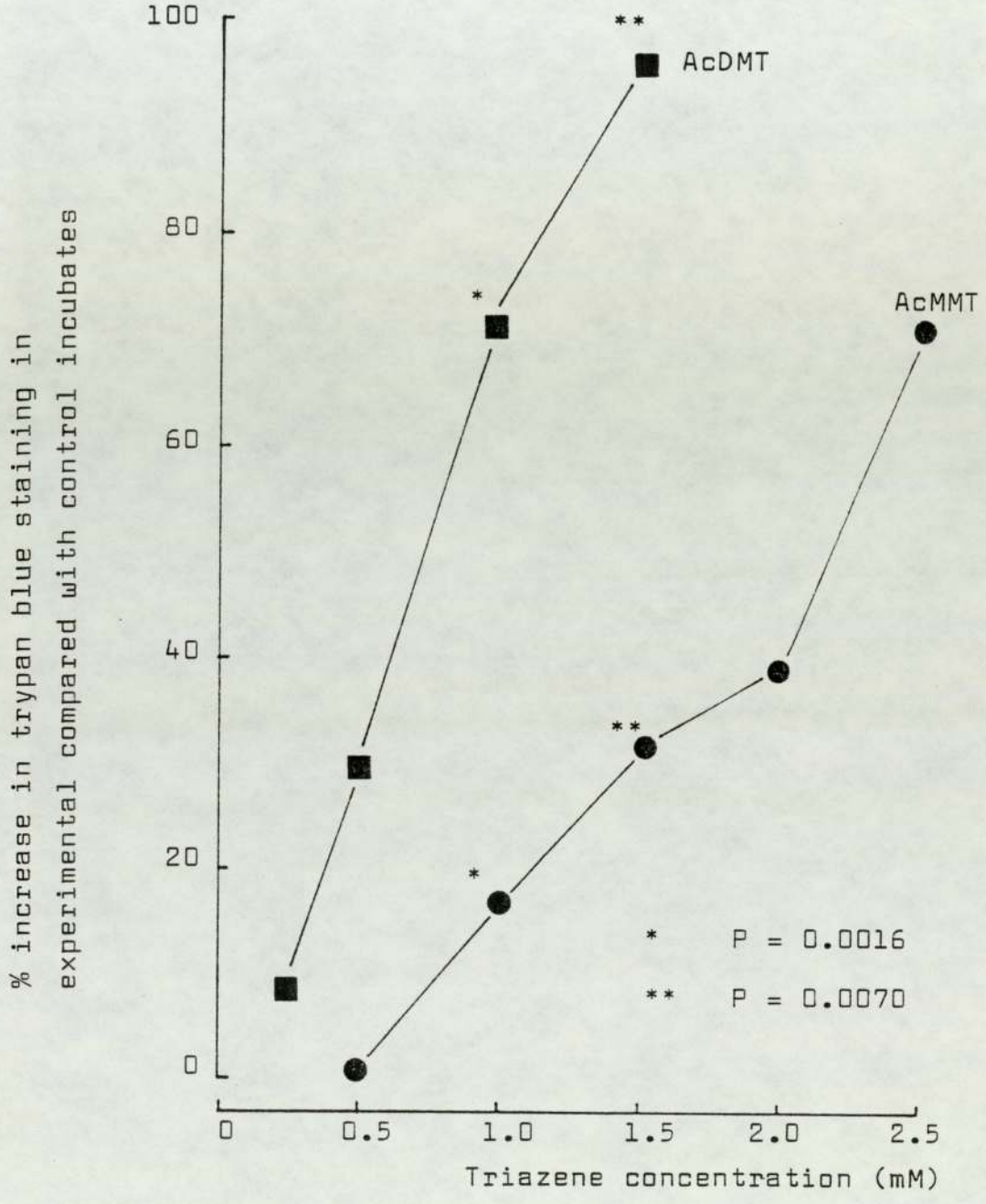


Figure 42 Toxicity of AcDMT and AcMMT to mouse hepatocytes after 2 hours incubation

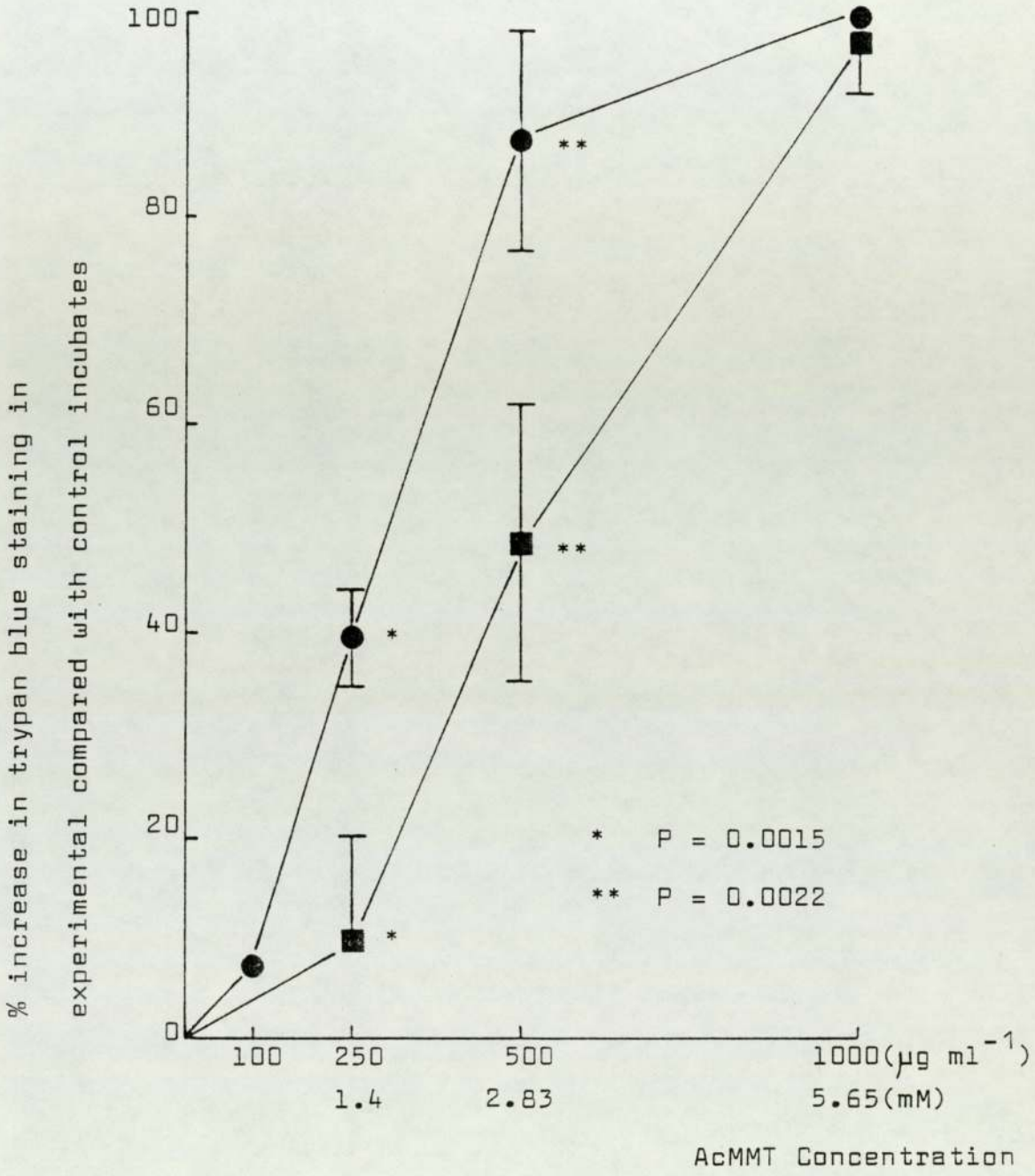


Figure 43 Toxicity of AcMMT to TLX5 (●) and TLX5R (■) cells after 2 hours incubation

D.4 Glutathione studies

Glutathione levels were determined by comparison with a standard calibration curve obtained under experimental conditions (section C.4.1.3), where each point represents the mean of 4 determinations (see Appendix). Total glutathione concentrations are expressed in GSH equivalents (GSH + $\frac{1}{2}$ GSSG). In the presence of 2-vinylpyridine, reduced glutathione (GSH) values become zero, indicating complete derivatisation of the thiol group, whereas oxidised glutathione (GSSG) values were reduced to only 90% of the value obtained without 2-vinylpyridine.

D.4.1 Glutathione levels in TLX5 lymphoma ascites cells

Total glutathione levels were assayed in both triazene sensitive (TLX5) and resistant (TLX5R) cell lines. The results are shown in table 24. Oxidised glutathione levels were too low to be measurable.

D.4.2 The effect of triazenes on the glutathione levels in mouse hepatocytes

D.4.2.1 Interference by triazenes in the assay of a standard GSH solution

The first study was conducted to ascertain that triazenes do not interfere with the assay of a standard solution of GSH (50 μM) in 10% metaphosphoric acid (section C.4.3). The results are shown in table 25 for a range of concentrations of AcDMT (Ij) and AcMMT (Ik). All of the glutathione detected by the assay in the presence of AcDMT was in the oxidised form. Figure 44 shows the increasing GSSG concentrations as a percentage of total glutathione in the presence of increasing concentrations of AcMMT.

Table 24 Total glutathione levels in TLX5 lymphoma ascites cells

Cell line	Total glutathione (nmol/10^6 cells)
TLX5	0.655 ± 0.27
TLX5R	0.739 ± 0.33

Table 25 Assay of a standard GSH solution (50μM) in the presence of AcDMT or AcMMT

Triazene	Triazene concentration (μM)	GSH assay value as % of control without triazene
AcDMT (Ij)	100	18.2
	200	5.0
	250	7.2
AcMMT (Ik)	100	67.5 ± 4.4 *
	200	67.0 ± 10.8*
	300	65.1
	400	59.0

* mean and standard deviation of at least 3 determinations

D.4.2.2 Influence of AcMMT on hepatocyte glutathione levels

From nine separate mouse hepatocyte preparations, total glutathione levels were determined as 13.02 ± 2.87 nmol/10^6 cells. The majority of the glutathione was in the reduced form, with the concentration of GSSG as a percentage of the total glutathione concentration 5.94 ± 2.4%. Hepatocyte viability as assessed by trypan blue staining was greater than 70%.

The concentrations of AcMMT employed in this study (not greater than 500 μM), did not decrease hepatocyte viability below that of untreated control cells. The hepatocytes in control incubations showed a variable decrease in viability over 80 minutes, down to about 50% viability.

Total glutathione levels even in control cells decreased over the 80 minute incubation and to a variable extent, hence triazene treated cells were compared with control cells of the same cell preparations. At AcMMT concentrations of 200 μM and below a paired T-test was used for statistical analysis. The lowest concentrations of AcMMT employed, 20 and 50 μM, had no effect on hepatocyte glutathione levels. At AcMMT concentrations of 100, 200 and 500 μM, decreased total glutathione levels were seen in triazene treated cells (figures 45 and 46). The decrease in glutathione concentration was significant after 40 minutes incubation with AcMMT 200 μM (Paired T-test, $P < 0.036$) and after 80 minutes with a concentration of 500 μM ($P = 0.0109$).

There was no increase in oxidised glutathione as a percentage of total glutathione in triazene treated cells as compared with control cells after 80 minutes. In only a very few control incubations did oxidised glutathione levels increase at all, and then by only a very small extent.

Total glutathione levels in the supernatant after spinning the hepatocytes down are shown in table 26.

D.4.3 The effect of 1-(4-acetylphenyl)-3,3-dimethyltriazene (AcDMT) (Ij) and 1-(4-acetylphenyl)-3-methyltriazene (AcMMT)(Ik) on the activity of glutathione reductase in mouse hepatocytes

Mouse hepatocytes were incubated with AcDMT or AcMMT at concentrations of 200 and 500 μM for up to 80 minutes. The effect of the triazenes on glutathione reductase activity compared with zero time values is shown in figure 47. BCNU at a concentration of 50 μM was used as a positive control. Neither triazene nor BCNU treatment caused any decrease in cell viability as measured by trypan blue staining when compared with control untreated cells.

D.4.4 The effect of triazenes on glutathione oxidation in mouse liver homogenate

Glutathione (GSH) oxidation was determined by measuring the increase in GSSG as a percentage of the total glutathione in GSH equivalents (GSH + $\frac{1}{2}$ GSSG) with time.

The rate of oxidation of GSH in an incubate of liver homogenate in Earl's buffer (pH 7.4) is shown in figure 48. This is the control rate to which all other experimental rates were compared. The total glutathione

Table 26 Total glutathione concentrations in hepatocyte supernatant from control and AcMMT treated cells

Sample	Time of incubation (min)	Total glutathione concentration (μM)
control	0	14.16 ± 4.3
control	80	17.05 ± 9.4
100 μM AcMMT	80	25.3
200 μM AcMMT	80	25.0 ± 10.5
control (n = 2)	0	8.69
control	80	10.95
500 μM AcMMT	80	13.66

concentration of 154.7 ± 22.3 μM was measurable by the assay. The percentage of GSSG at time 0 was always very low; ~ 3.0% of the total glutathione.

On the same figure is shown the lack of GSH oxidation in similar liver preparations, but incubated on ice, or at 37°C under a nitrogen atmosphere. Where the liver homogenate was put into 0.25M sucrose for incubation rather than Earl's buffer, a significantly lower degree of oxidation was seen after 20 minutes (P = 0.0012) (figure 48). Also shown on figure 48 is the effect of heating the liver homogenate at 90°C for 15 minutes before incubating as in the control system. A significantly lesser oxidation was again seen after 20 minutes (P = 0.0002).

No difference was seen in the GSH oxidation rate between control livers and livers perfused with 0.25M sucrose prior to homogenate preparation (figure 49).

The extent of GSH (150 μM) made up in Earl's buffer pH 7.4, and incubated at 0°C or 37°C is shown in table 27. For comparison, the GSSG levels as a percentage of total glutathione in liver homogenate incubated in Earl's buffer at 37°C are shown. The GSSG as a percentage of total glutathione at 20 minutes (at 37°C) was significantly different (P = 0.0077) between incubates with and without liver homogenate.

Addition of AcDMT (Ij) to the liver homogenate preparation at a concentration of 1mM resulted in a decrease of the total glutathione assay value to 25% of the level without triazene, and of the remaining free glutathione, 35%

Table 27 GSH oxidation in Earl's buffer (pH 7.4)

Incubation conditions	GSSG as % of total glutathione		
	0 min	10 min	20 min
Earl's buffer 0°C (n = 2)	3.79	3.65	4.19
Earl's buffer 37°C	5.98 ± 2.8	15.58 ± 11.0	34.5* ± 15.0
Liver homogenate in Earl's buffer 37°C	2.47 ± 1.7	2.92 ± 12.6	71.1* ± 20.0

* P = 0.0077

Table 28 Liver homogenate GSH oxidation at 37°C in the presence of AcMMT

AcMMT(Ik) concentration (mM)	GSSG as % of total glutathione		
	0 min	10 min	20 min
0	2.47 ± 1.7	22.92 ± 12.6	71.1* ± 20.0
0.25 (n = 1)	4.0	32.6	70.8
0.5 (n = 1)	3.5	25.5	47.2
1.0 (n = 3)	3.70 ± 2.2	9.96 ± 6.6	25.77* ± 21.9

* P = 0.0024

was in the oxidised form. AcMMT at the same concentration (1mM) did not interfere with either the time zero total glutathione assay value or the degree of GSH oxidation in the liver homogenate preparation. The effect of AcMMT (Ik) at concentrations 0.25 - 1mM on the rate of GSH oxidation in the liver homogenate is shown in table 28. At a concentration of 1 mM, there was significantly less GSSG at 20 minutes than in control incubates without AcMMT ($P = 0.0024$).

The effect of other monomethyltriazenes (CyMMT; Ir, Ig, Iy) and a monoethyltriazene AcMET (In) on glutathione oxidation are shown in figure 50, and of two arylamines (Xa, Xc) in figure 51.

The effect of varying concentrations of EDTA on the oxidation of glutathione in the liver homogenate system is shown in table 29. At a concentration of 15 mM, a significantly lower amount of GSSG as a percentage of total glutathione was found after 20 minutes when compared with control incubates ($P = 0.0015$).

Diethyldithiocarbamic acid had no effect on glutathione oxidation rate at concentrations of 1 mM and 10 mM, neither did sodium azide 1 mM.

Table 29 Liver homogenate GSH oxidation at 37°C in the presence of EDTA

EDTA concentration (mM)	GSSG as % of total glutathione		
	0 min	10 min	20 min
0	2.47 ± 1.7	22.92 ± 12.6	71.10* ± 20.0
2.5 (n = 2)	2.00	36.10	61.05
5.0 (n = 2)	2.57	18.21	65.18
10.0 (n = 2)	1.96	20.12	44.29
15.0 (n = 4)	1.62 ± 1.2	19.13 ± 4.7	25.04* ± 8.8

* P = 0.0015

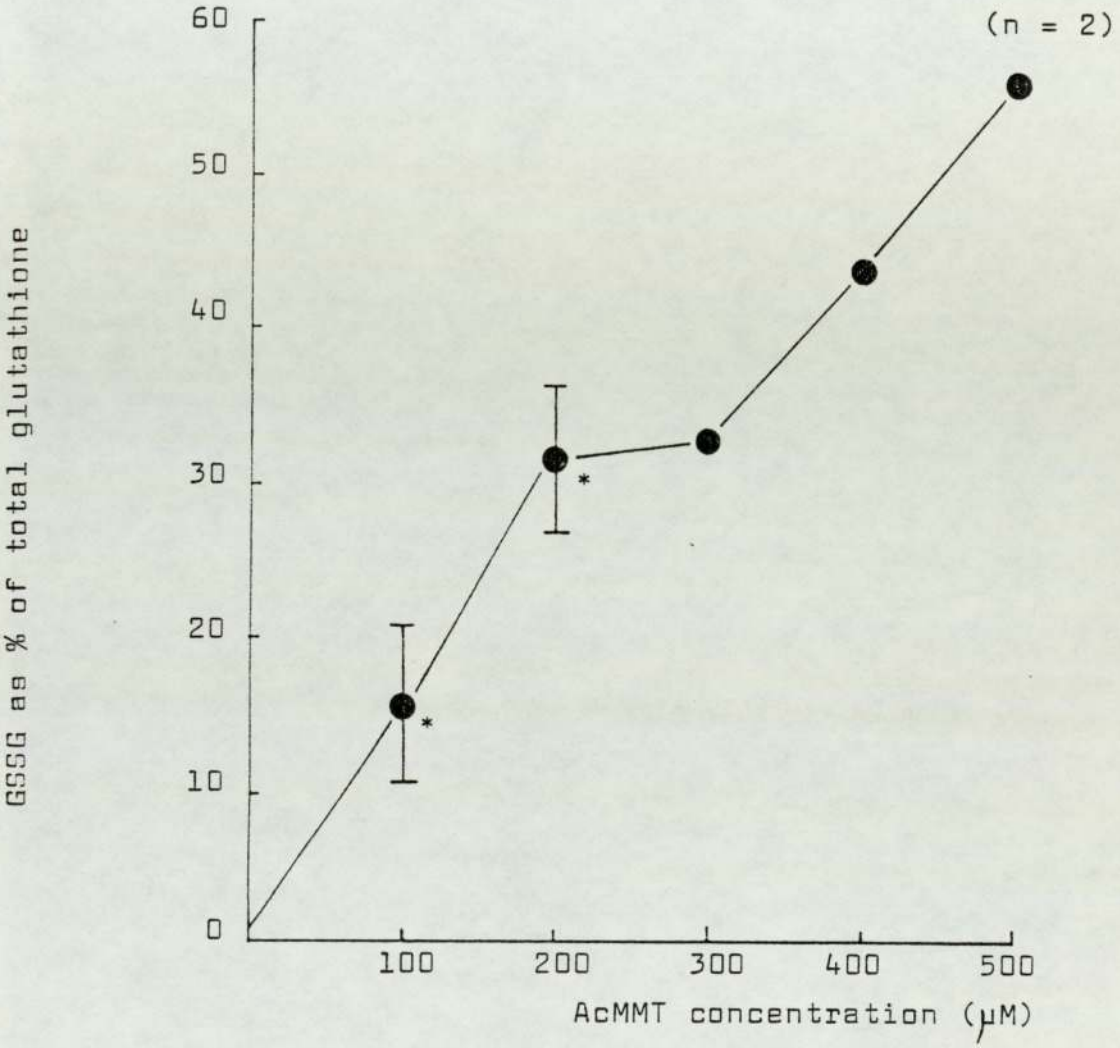


Figure 44 Oxidation of free GSH to GSSG in 10% metaphosphoric acid upon addition of AcMMT

* mean and standard deviation of at least 3 determinations

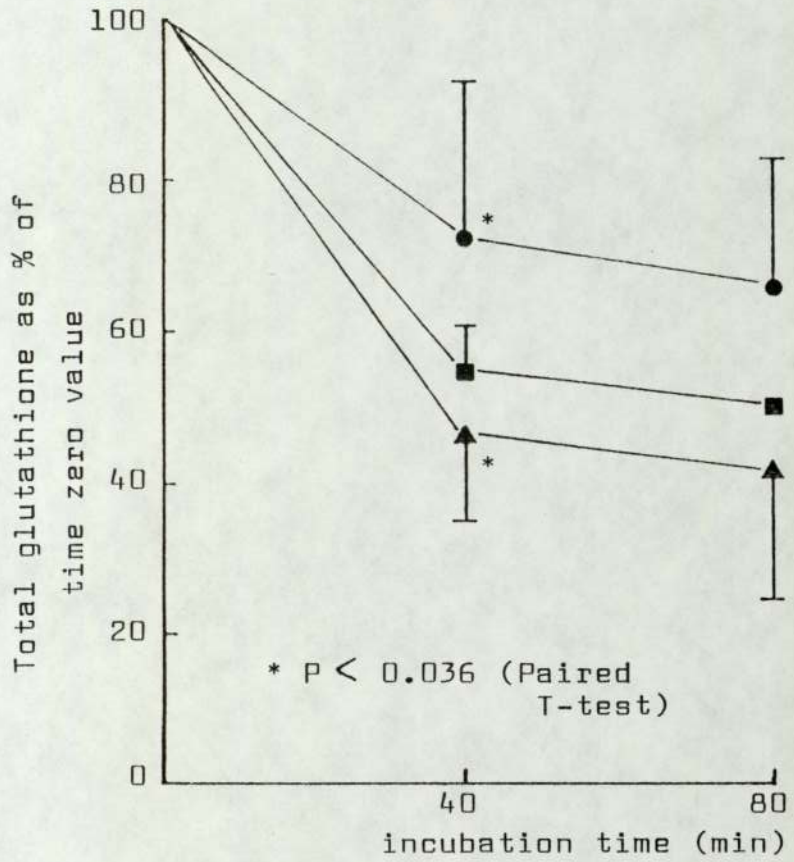


Figure 45 Decrease in total glutathione levels in mouse hepatocytes after incubation with AcMMT 100 μM (■) and 200 μM (▲) (● - control incubation)

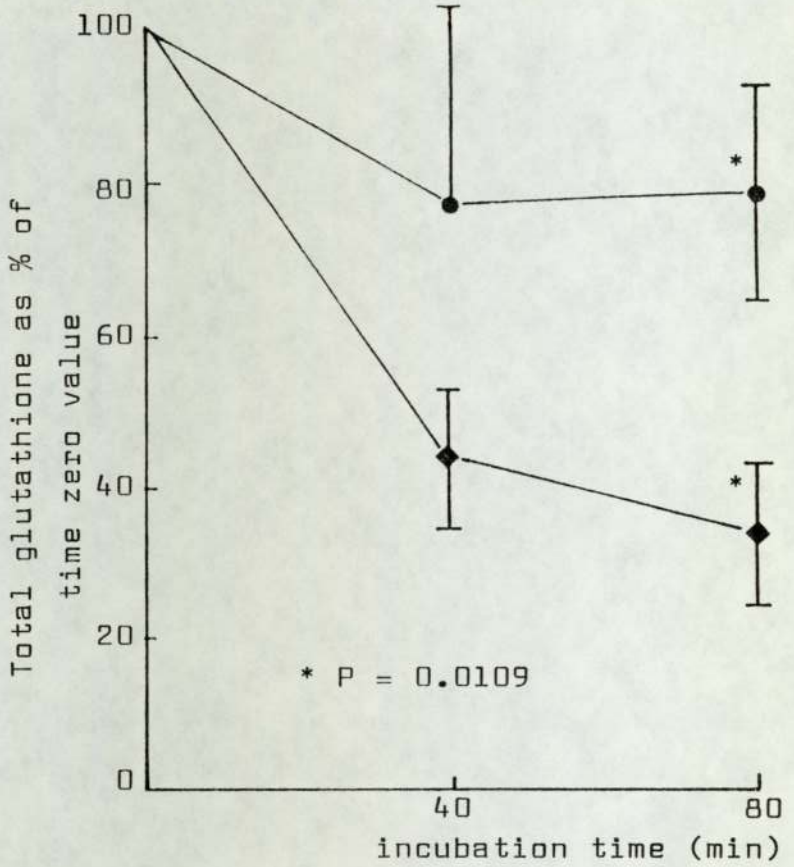


Figure 46 Decrease in total glutathione levels in mouse hepatocytes after incubation with AcMMT 500 μM (◆) (● - control incubation)

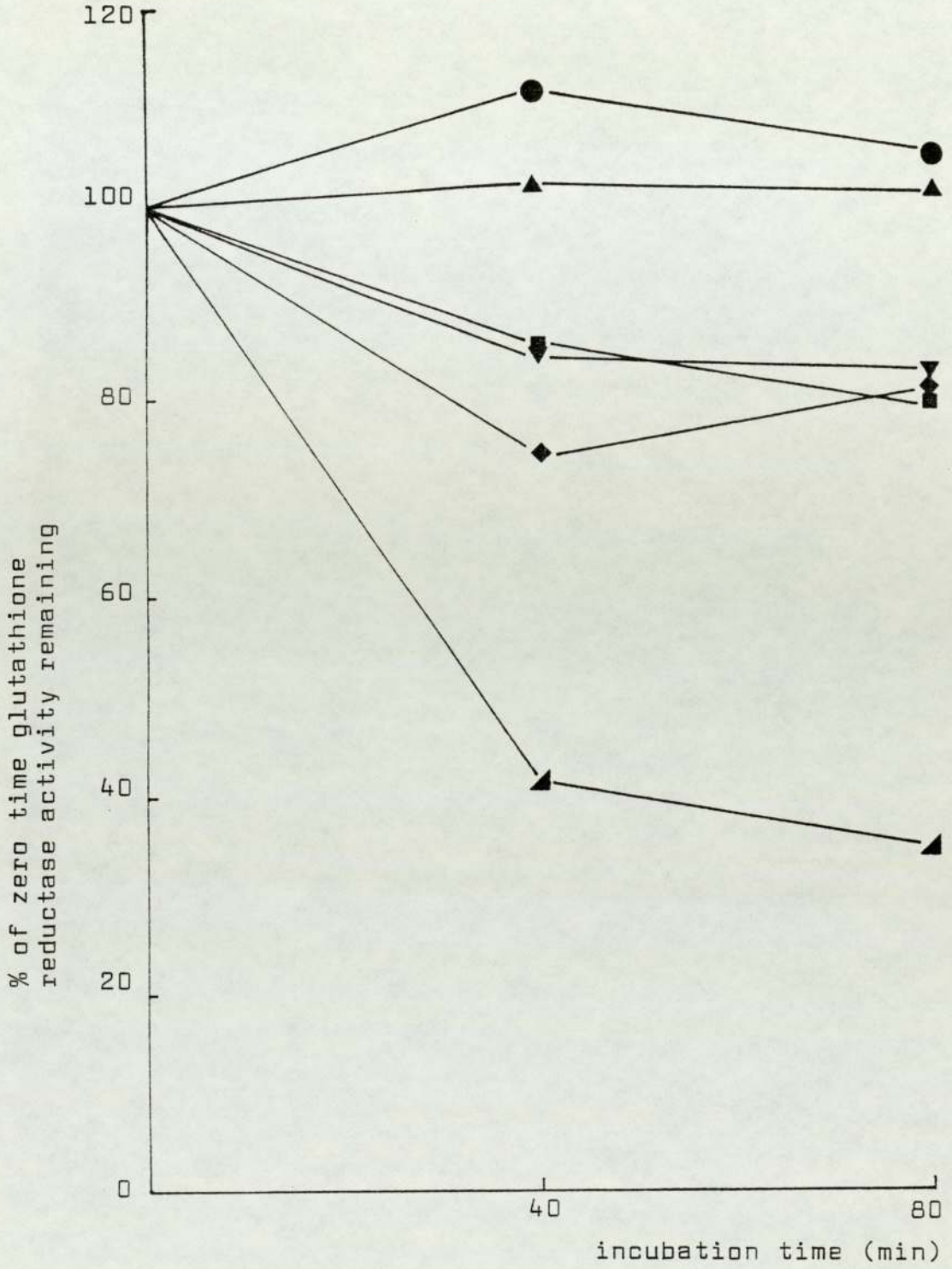


Figure 47 The effect of AcDMT, AcMMT and BCNU on glutathione reductase activity in mouse hepatocytes (n = 2)

AcDMT 200 μM (◆) AcDMT 500 μM (■)

AcMMT 200 μM (▲) AcMMT 500 μM (▼)

BCNU 50 μM (◢) Control incubation (●)

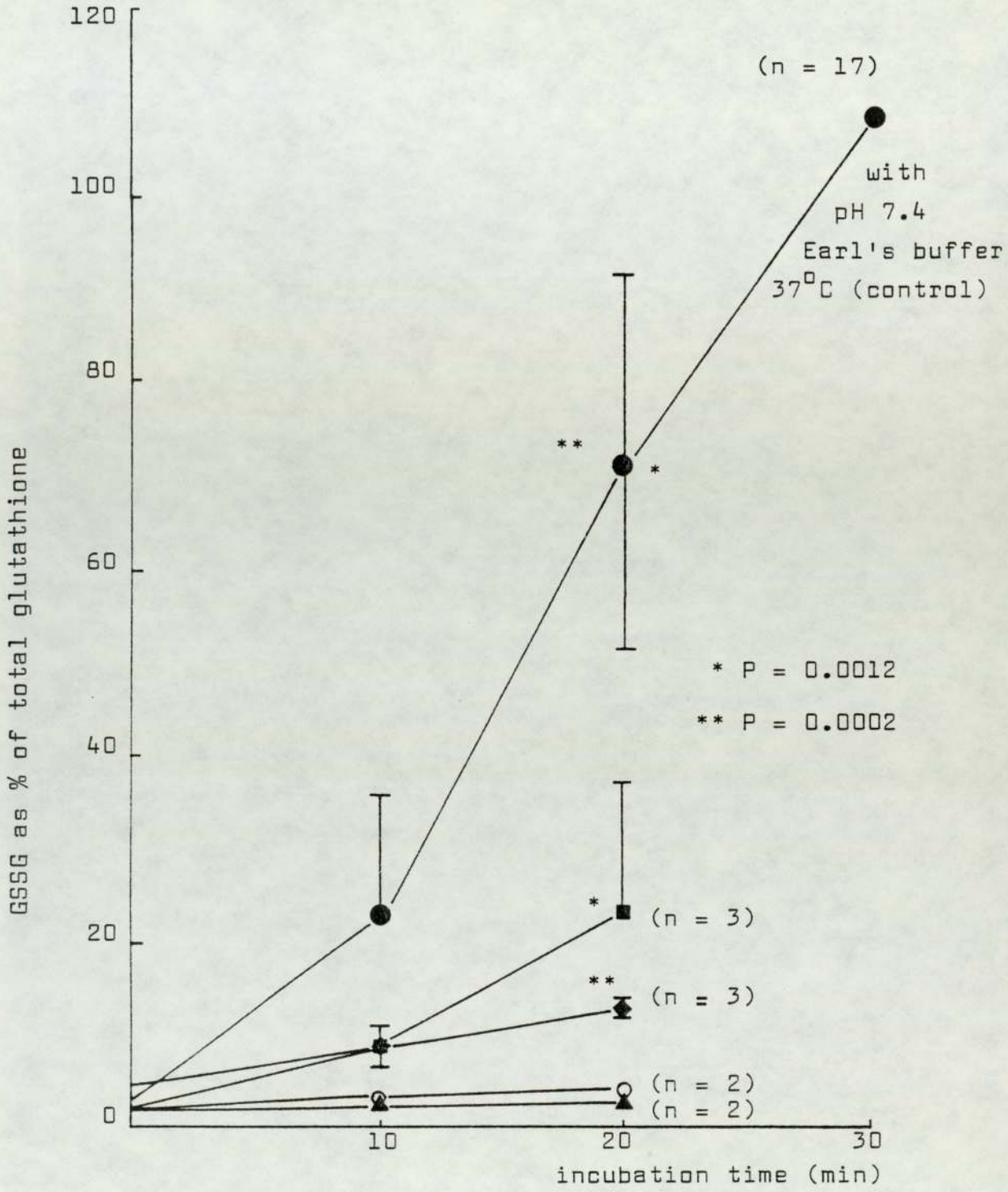


Figure 48 GSH oxidation to GSSG in mouse liver homogenate under different incubation conditions.
(●) control, (■) with sucrose 37°C,
(◆) heat inactivated liver 37°C, (○) with Earl's buffer on ice (▲) with Earl's buffer under nitrogen.

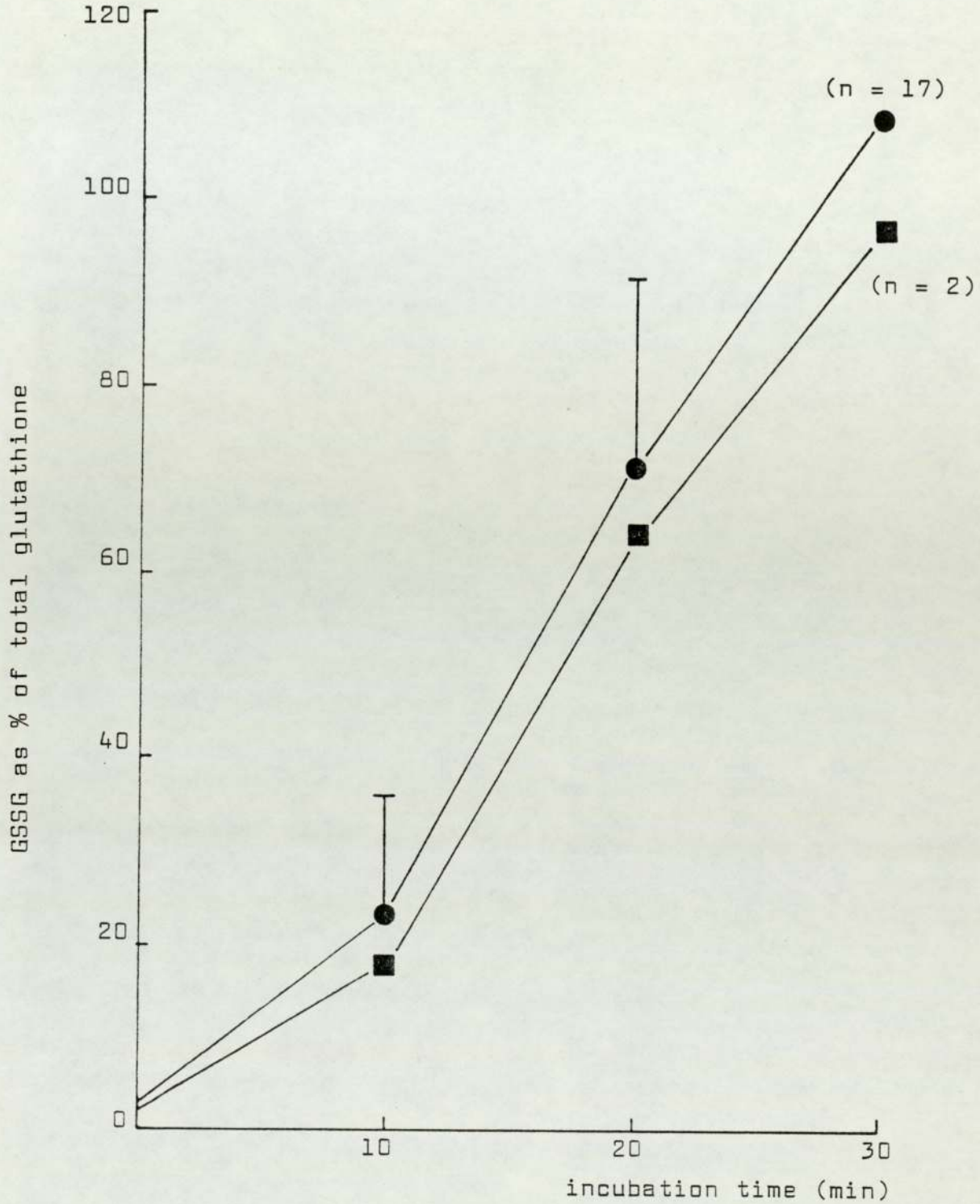


Figure 49 GSH oxidation to GSSG in mouse liver homogenate incubated in pH 7.4 Earl's buffer at 37°C.(●) control, (■) liver perfused with sucrose prior to homogenate preparation.

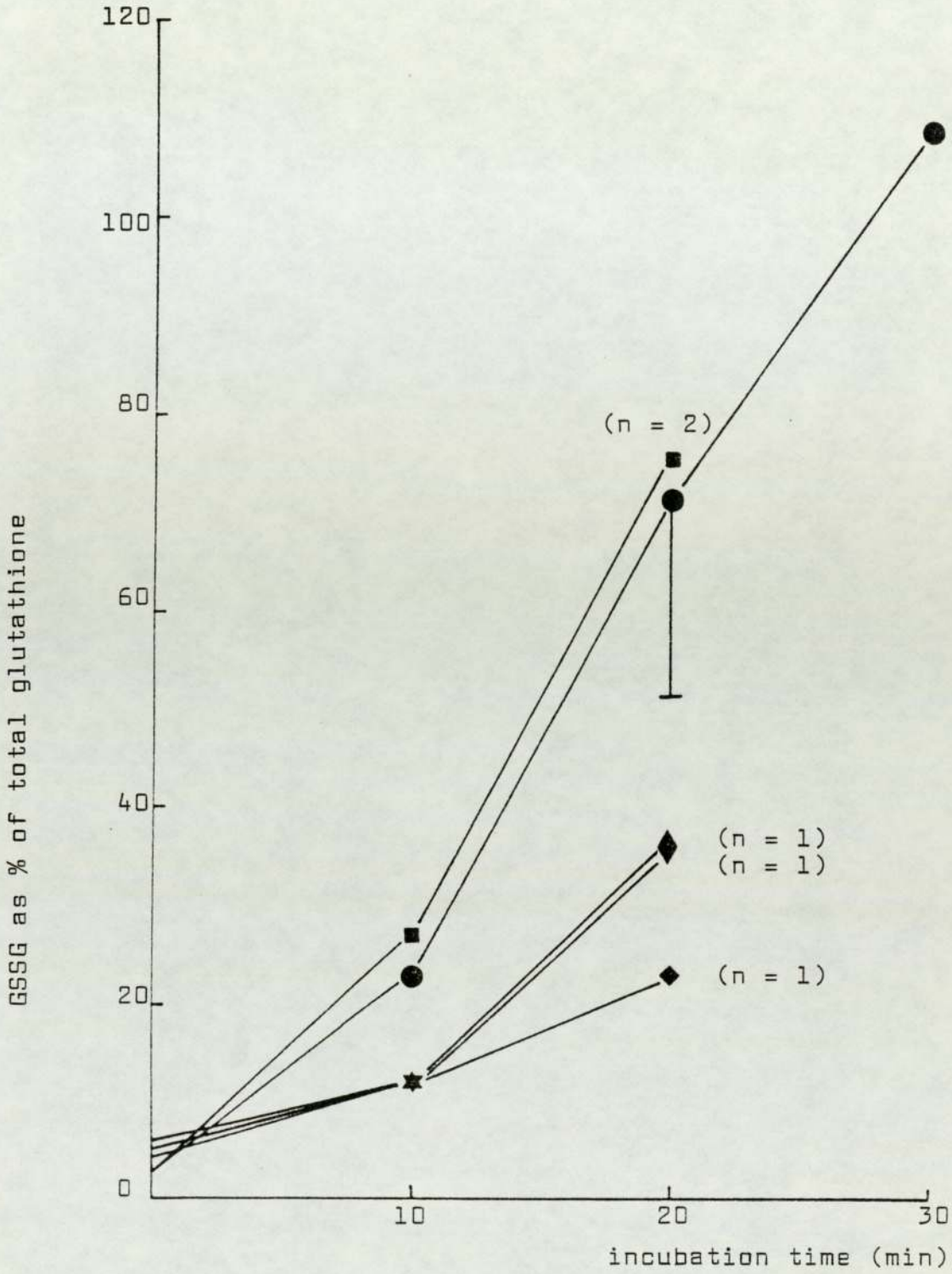


Figure 50 GSH oxidation to GSSG in mouse liver homogenate incubated in pH 7.4 Earl's buffer at 37°C in the presence of monoalkyltriazenes. (●) control, (■) CyMMT 1 mM, (▲)(Iy) 1 mM, (▼) AcMET 1 mM, (◆) (Ig)1 mM

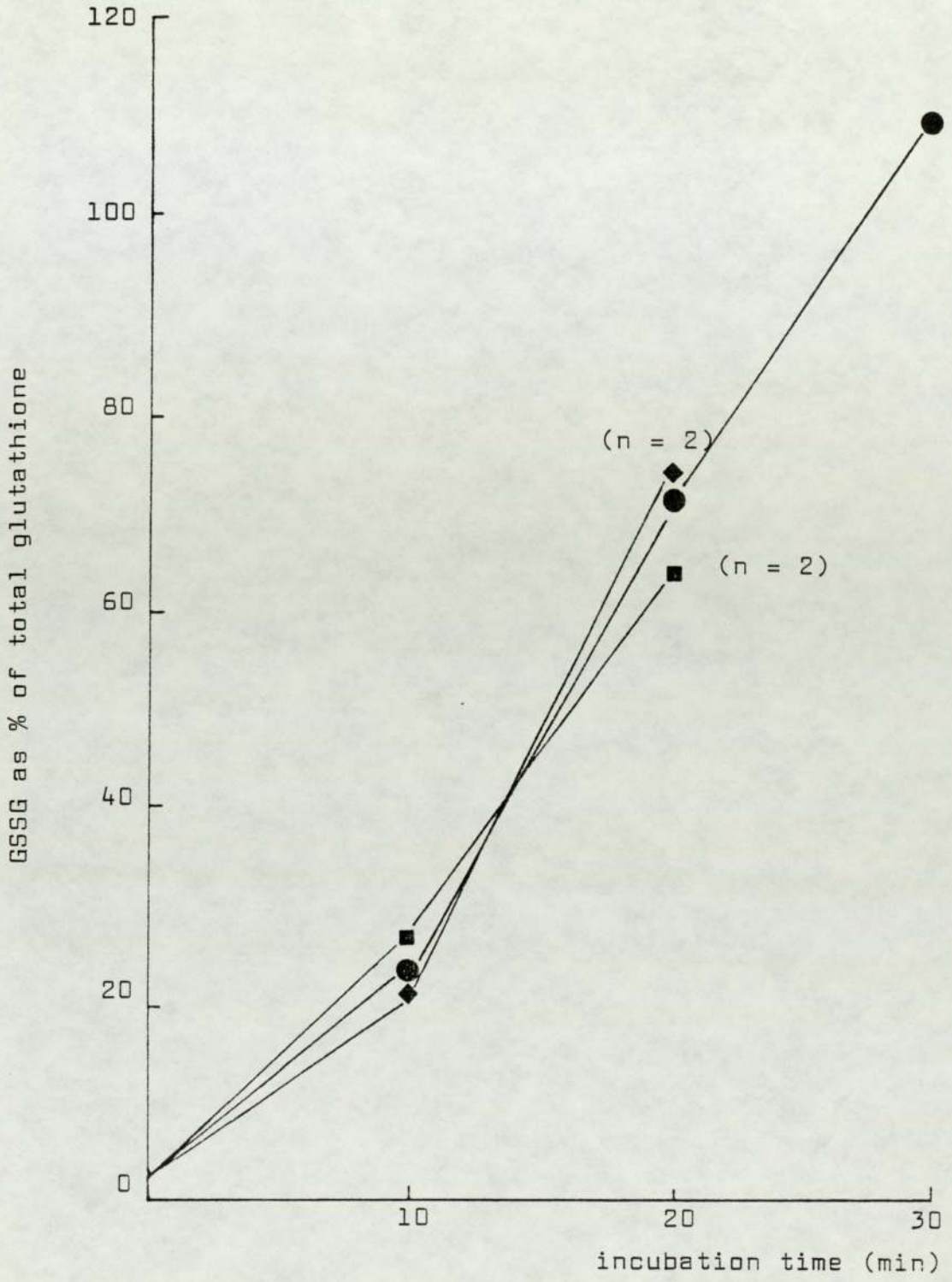


Figure 51 GSH oxidation to GSSG in mouse liver homogenate incubated in pH 7.4 Earl's buffer at 37°C in the presence of arylamines. (●) control, (◆) (Xc) 1 mM, (■) (Xa) 1 mM

PART E

DISCUSSION

PART E DISCUSSION

E.1 Photodecomposition of DTIC

Many of the cytotoxic drugs currently used clinically, for example DTIC, cyclophosphamide, bleomycin and cisplatin, are prone to chemical decomposition in solution and are supplied as powders to be reconstituted just prior to parenteral administration. Shealy and co-workers have shown pure DTIC to be stable in phosphate buffer pH 7.4 for at least 24 hours, provided solutions were kept in the dark, or exposed only to fluorescent light (11, 14). This result was confirmed in the present study (table 7). However exposure of a DTIC solution at pH 7.4 to sunlight resulted in dissociation of the molecule to yield dimethylamine and diazo-IC, and thence 2AH (11). In contrast, photolysis of an aqueous solution of the clinical formulation, DTIC-Dome (Dacarbazine), which contains DTIC as the citrate salt and mannitol, yields not 2AH but 4-carbamoylimidazolium-5-olate (OIC) (IXa) (144, 145), a product spectroscopically distinct from 2AH (table 6, figure 52).

The present work revealed that the influence of pH on the photodecomposition pathway is responsible for the differences between the results reported by Shealy using pure DTIC at pH 7.4 and those upon photolysis of DTIC-Dome in water where the pH is between 3 and 4. In the present study, 2AH was formed on decomposition of both pure DTIC and DTIC-Dome at pH 7.4 (table 7). Evidently the citric acid and the mannitol in the formulated product do not influence decomposition. Citric acid produces an acidic solution in water, but

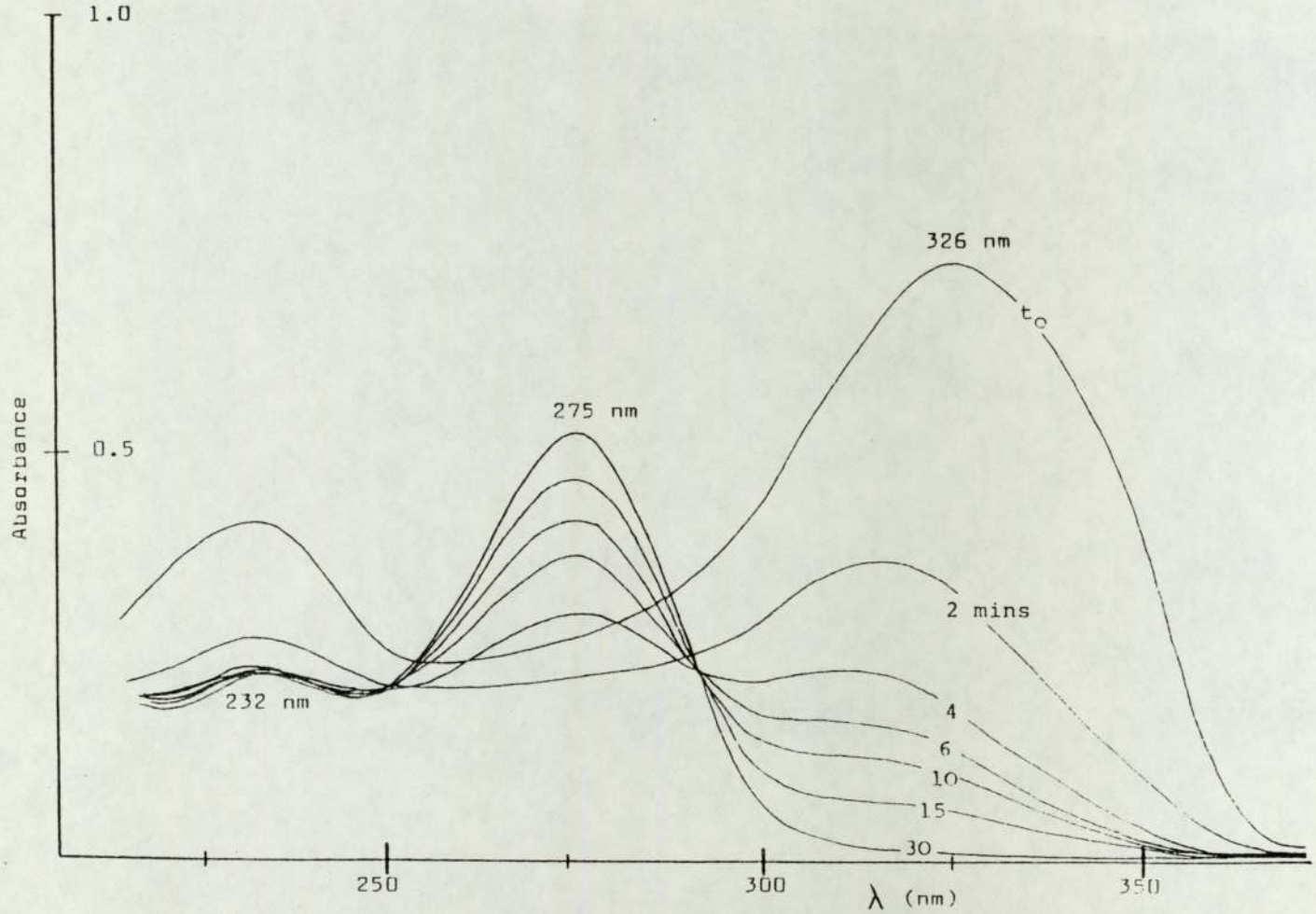


Figure 52 Photodecomposition of DTIC-Dome in water at 20°C.

Spectra were recorded at the time intervals indicated.

DTIC-Dome and pure DTIC yielded the same photoproducts in buffer over the pH range 1 - 12.

Photolysis of diazo-IC in the presence of citric acid, but not mannitol, also afforded OIC (144). The fact that DTIC and diazo-IC yielded identical products under the same pH conditions (table 7) suggests that the latter is an intermediate in the purely photochemical conversion of DTIC to OIC. A mechanistic interpretation is presented in figure 53 (146).

At pH 1, the UV spectrum of diazo-IC exhibits a substantial hypsochromic shift as compared with pH 2.5 (table 6). Diazo-IC, at this pH is presumed to exist as the protonated imidazolediazonium ion (XII) which affords 2AH by intramolecular cyclization. At alkaline pH ($>$ pH 7.4), 2AH is also formed, probably via the imidazolediazohydroxide (XIII). These two pathways occur in the dark and are not influenced by sunlight. In the intermediate pH range, 2 - 6, diazo-IC exists as a zwitterion; either diazonium (Va,Vb) or diazo (Vc) forms are possible. The photodecomposition is proposed to involve a carbene intermediate (XIV) generated from the diazo species (Vc); this carbene is then quenched by water to afford OIC.

OIC is the aglycone of the antibiotic bredinin (IXb) isolated from cultures of Eupenicillium brefeldianum M-1266 (147). Bredinin has been shown to inhibit the growth of L5178Y cells (147, 150), other mammalian cell lines (150), and also to have immunosuppressive activity (150, 151). OIC is itself cytotoxic to L5178Y cells (148). If a decomposition route to OIC were to exist in vivo, it is possible that bredinin may play a rôle in the cytotoxicity of DTIC, since a salvage

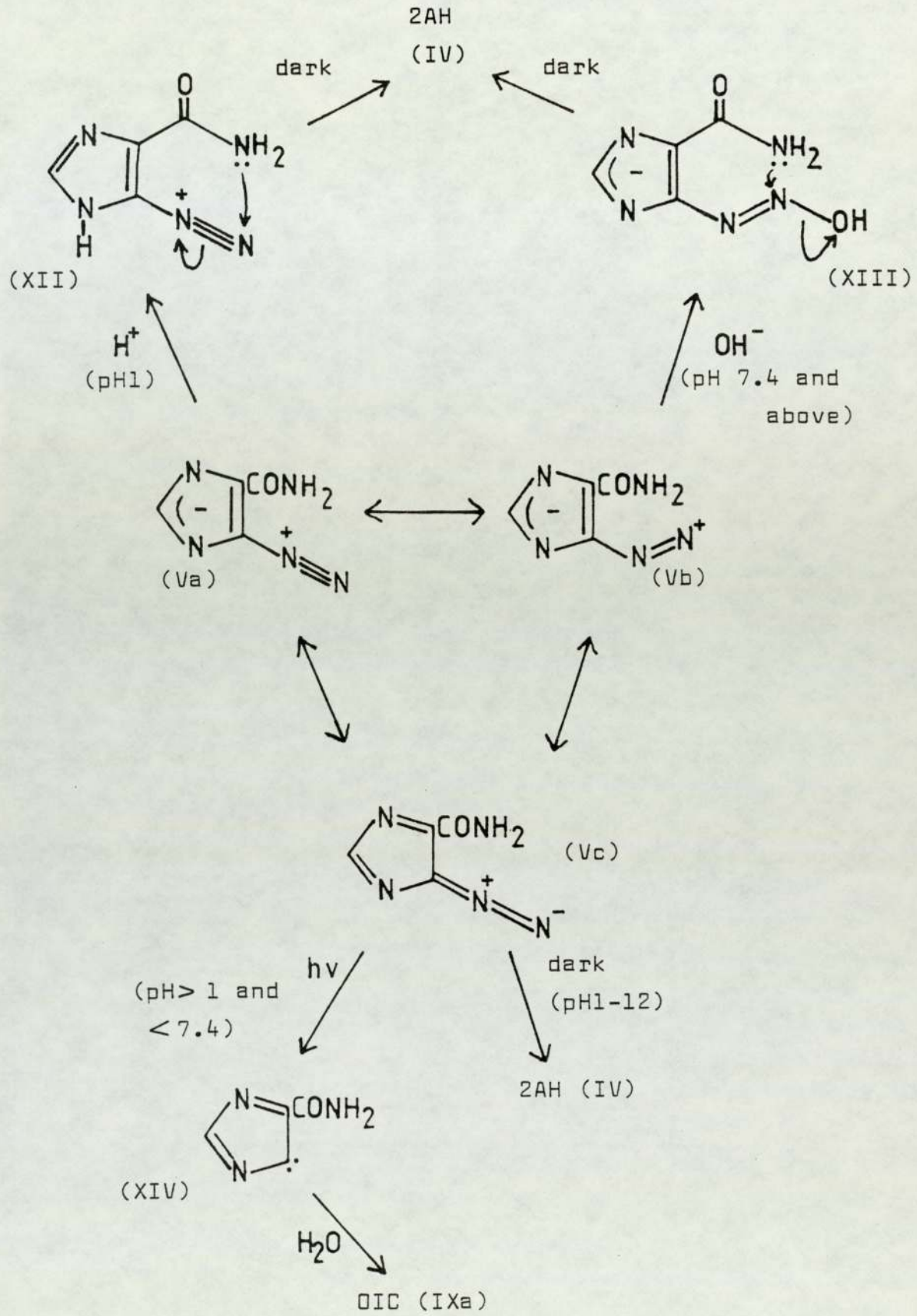
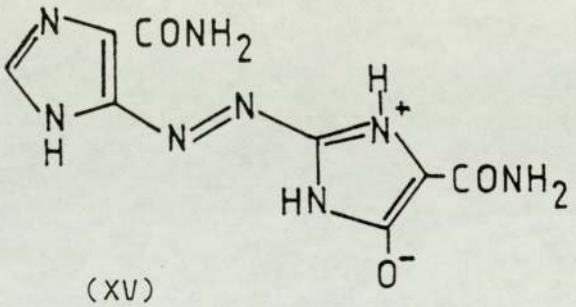


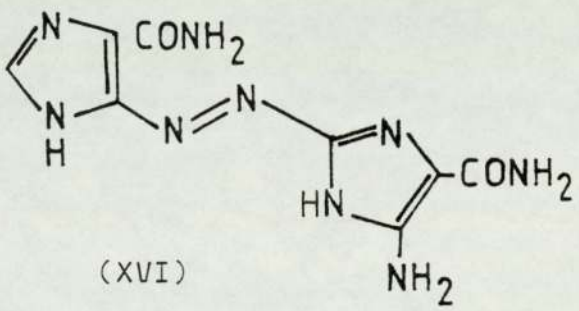
Figure 53 Decomposition of diazo-IC under different conditions.

pathway has been identified in mammalian cells whereby bredinin can be synthesized from its aglycone precursor (152).

DTIC was most photo-stable under mildly acidic conditions. For diazo-IC, the pH of greatest stability in the dark was pH 3 (table 8). Thus for both decomposition routes of DTIC, greatest stability was seen within the pH range of the formulated DTIC-Dome in water.



(XV)



(XVI)

The maroon precipitate produced on photodecomposition of high concentration DTIC-Dome in water has previously been identified as 4-carbamoyl-2-(4-carbamoylimidazol-5-ylazo) imidazolium-5-olate (XV) (144), formed from coupling of

diazo-IC with OIC. This highly coloured dye is related to the aminoimidazoazoimidazole (XVI) formed in solutions of DTIC-Dome stored at elevated temperatures in the dark (145). The spectroscopical identification of 2AH in the filtrate after removal of the precipitate (XV) formed during photolysis of DTIC-Dome was unexpected considering the starting pH of photodecomposition (3 - 4), which remained stable. It would seem contrary to the mechanism of figure 53 which predicts the photoproduct of DTIC to be OIC at mildly acidic pH. A likely explanation is that DTIC decomposes to yield diazo-IC which then couples with the OIC formed concurrently to produce the azo-dye (XV). This results in development of colour in the solution, and further decomposition of diazo-IC is a 'dark' reaction which, as predicted, yields 2AH.

The implications for patients receiving any of the photoproducts of DTIC generated during preparation or administration of the drug are unknown. It would seem prudent to protect infusions from light at all times since there has been a suggestion that some of the side-effects of DTIC, particularly local venous pain at the injection site, are due to photodegredation products of the drug (153). A new delivery system for administering cytotoxic drugs protecting them at all times from the light has recently been developed (153). There is some evidence that there is reduced toxicity when this system is used with DTIC (155).

E.2 Interaction between DTIC and nucleophiles in the presence of sunlight

DTIC is cytotoxic *in vitro* against *B.subtilis* and *E.coli* B particularly in the presence of sunlight (59, 60). L-cysteine and glutathione can reverse this inhibitory activity, and also that of diazo-IC, possibly by interaction between the diazo species and thiol groups. An interaction was confirmed in this study for L-cysteine by the appearance of a peak of λ max 340 nm (section D.1.2.1), and the proposed formation of an azothioether is shown in figure 54. Further characterization of the coupled product was not attempted.

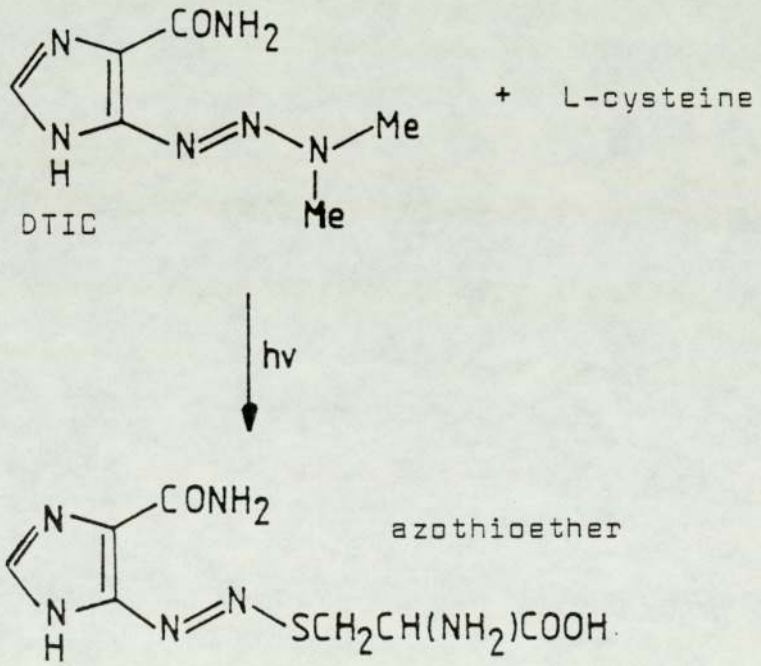


Figure 54 Proposed interaction between DTIC and L-cysteine.

DTIC used in the treatment of malignant melanoma is frequently only one component of a combination regime.

A survey of Birmingham hospitals revealed that, on occasions, two cytotoxic drugs are mixed during administration. If the combinations are not protected at all times from light, reactive species generated from DTIC may interact with other drugs. There was, however, no detectable interaction with any of the cytotoxic agents used in the present study (table 9), despite the high chemical reactivity of DTIC photoproducts, particularly diazo-IC. The absence of interaction was particularly unexpected for the thiol-containing cytotoxic drug 6-mercaptopurine, since interaction has been shown between DTIC and the thiol groups of other compounds in the presence of light (section D.1.2.1).

As interaction with diazo-IC is responsible for the formation of azo-dyes described in section D.1.2.3 for NEDA and 2-naphthol. A mixture of diazo-IC and 2-naphthol yielded a scarlet red product with λ max 496 nm (figure 55). The coupling reaction has been used in a colorimetric determination of DTIC (156) based on the reaction between diazo-IC and NEDA [Bratton-Marshall reagent (157)]. The same type of colour reaction with formation of an azo-dye has also been used in tlc spray detection for triazenes (50, 158).

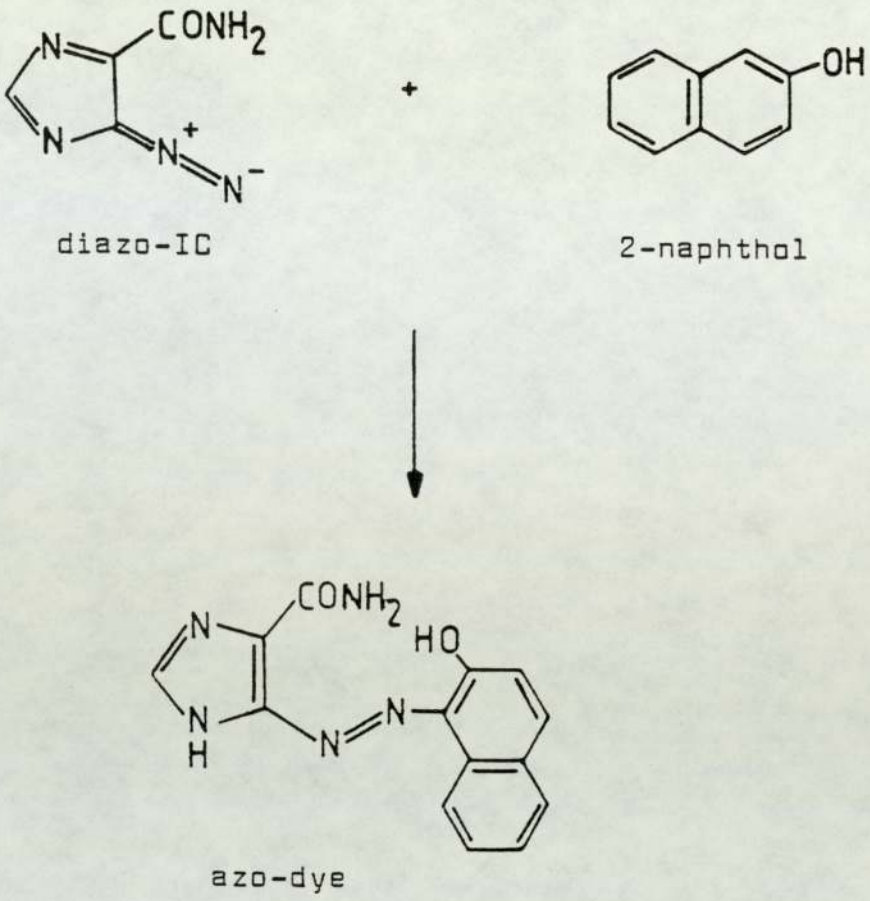
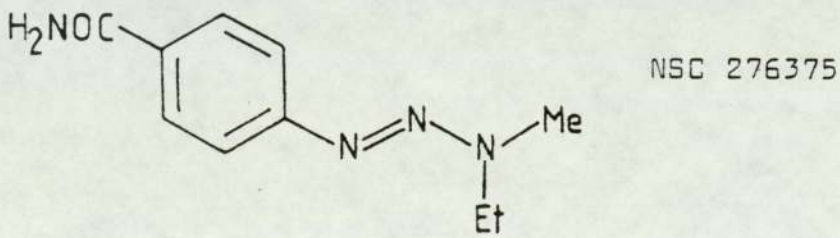


Figure 55 Interaction between diazo-IC and 2-naphthol

E.3 Stability of other triazene derivatives

E.3.1 AcDMT



NSC 276375

Initial studies of triazene antitumour properties showed that aryldimethyltriazenes were as active against

experimental tumours, such as mouse L1210 leukemia, as DTIC and other imidazolecarboxamides, but without the associated problem of light catalysed decomposition (15). The aryltriazenes have never been tested in man, but may possess good antitumour activity. One such derivative, NSC 276375, is currently a candidate for phase I studies organised by the National Cancer Institute. Aryldimethyltriazene photostability was confirmed for AcDMT in the present study (table 10). The same dimethyltriazene was subjected to protolysis at acidic pH below 5.2, with formation of dimethylamine and a diazonium species which subsequently decomposed to other products (figure 56).

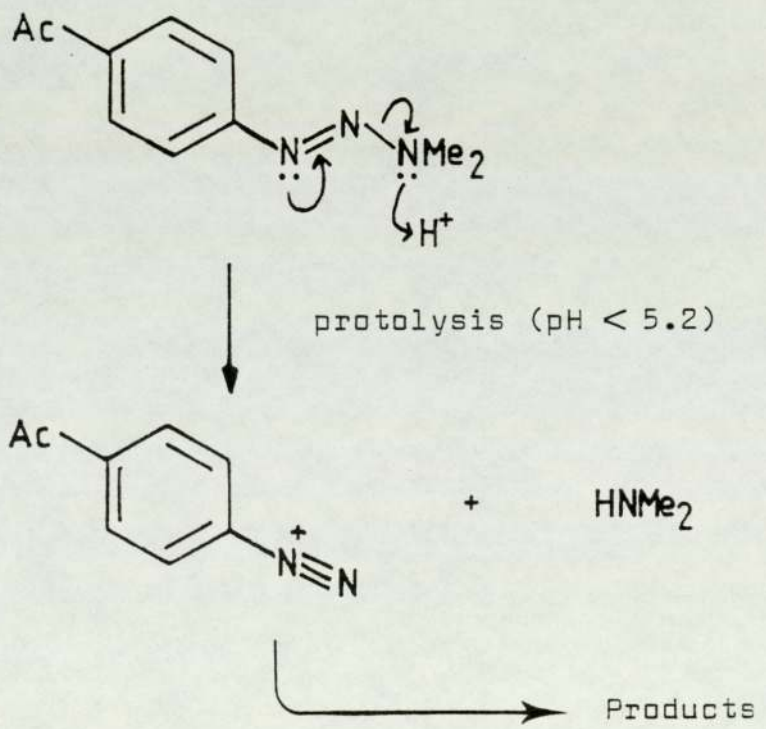


Figure 56 Protolysis of AcDMT

E.3.2 Monomethyl- and hydroxymethyl- triazenes

Activation of dimethyltriazenes by metabolism seems to be necessary for their antitumour activity, although this is difficult to ascertain due to the chemical instability of triazenes, particularly DTIC. Both monomethyl- and hydroxymethyl- triazenes have been proposed as the active antitumour species (41,46).

Arylmonomethyltriazenes have been shown to decompose in aqueous solutions at physiological pH in a way which can be described by first order kinetics (159). The half-life of AcMMT in phosphate buffer pH 7.4 was similar whether determined by UV-spectroscopy (13.8 min) or HPLC (15.1 min), but very different in buffers of different composition but identical pH (7.4) (15.1 - 66.6 min), as determined by HPLC (table 11). The decomposition of arylmonomethyltriazenes has been shown previously to exhibit a marked dependence on the composition of the medium, at least in aprotic solvents (160).

The half-lives of all the derivatives studied are longer than one minute (1.2 - 14.5 min) in phosphate buffer. Preussmann et al determined the half-life of phenylmethyltriazene (Ib) to be 3.5 minutes at 37°C in pH 7.4 buffer (41). The stabilising effect on monomethyltriazenes of serum added to the buffer solution has been demonstrated (41,161), and it was concluded that the stability of triazene (Ib) was great enough to allow distribution in vivo to distant tumour target sites.

That monomethyltriazenes have antitumour properties *in vivo* (45, 46), and also are carcinogenic and can give rise to tumours distant from the injection site (93), lends support to the view that they have a rôle as *in vivo* triazene transportable species. The dimethyltriazene (Iaa) is an active antitumour agent against TLX5 *in vivo* (50), despite the extreme instability of the corresponding monomethyltriazene (Ibb) ($t_{\frac{1}{2}}$, 1.2 min at 37°C in phosphate buffer, table 11). On the assumption that monomethyltriazenes are responsible for the antitumour activity of the dimethyltriazenes, this means that even the least stable monomethyltriazene is capable of reaching distant tumours from the site of its production in the liver.

There was very little difference between the half-lives of corresponding monomethyl- and hydroxymethyltriazenes (AcMMT and Il; Iy and Iz), and also the λ max of corresponding derivatives was identical (table 11). It is possible that the hydroxymethyltriazenes (Il, Iz) are extremely unstable, decomposing immediately in aqueous solution, so that the half-lives determined are actually those of the corresponding monomethyltriazenes (AcMMT), (Iy), the elimination products of the hydroxymethyltriazenes (figure 57). Alternatively, corresponding monomethyl- and hydroxymethyl- triazene derivatives may have identical λ max, but since the hydroxymethyltriazenes are very unstable first decomposing to yield the monomethyltriazenes, the spectroscopic method cannot distinguish between them. The antitumour properties of the hydroxymethyltriazene (Iz) are identical to those of the monomethyl derivative (Iy), in that it shows selectivity *in vivo*, but *in vitro* is equitoxic

to triazene sensitive and resistant TLX5 lines (51).

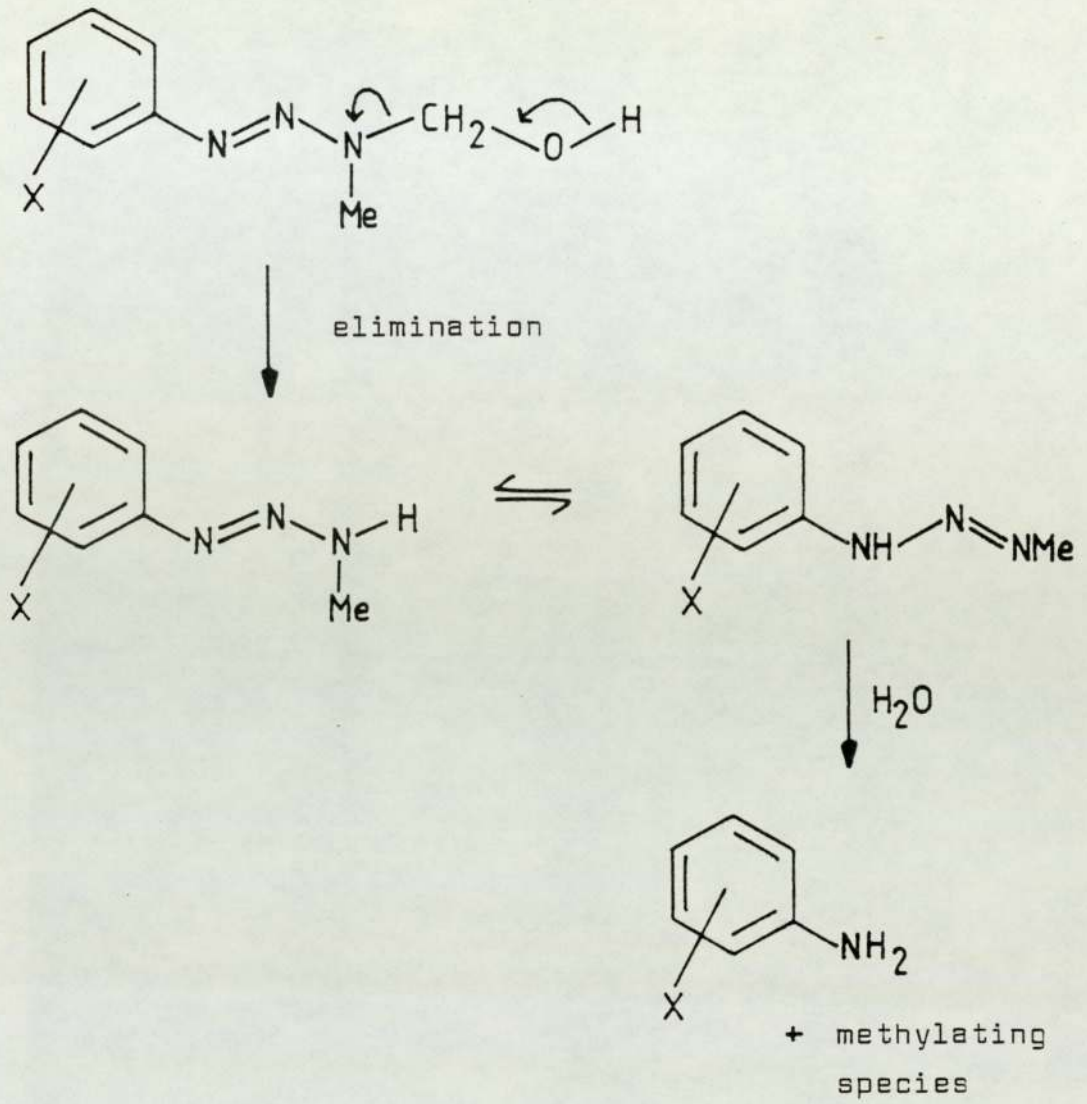


Figure 57 Degradation pathway for hydroxymethyltriazenes

Other N-methyl containing cytotoxic drugs such as hexamethylmelamine (162) and procarbazine (163) also yield hydroxymethyl derivatives on metabolism. The hydroxymethyl metabolite of hexamethylmelamine is relatively stable (only 2% decomposed after incubation for 16 minutes at 37°C in mouse whole liver homogenate) (164), and may be important in its mechanism of cytotoxicity. For the triazenes used in the present study, a similar rôle for the hydroxymethyl derivatives seems unlikely.

E.4 Preliminary investigations on the metabolism of AcDMT

In order to identify metabolites of AcDMT, a tlc system was developed on silica plates. This separated AcDMT, 4-aminoacetophenone (Xa) and the diaryltriazene (VIII), but the instability of AcMMT and the corresponding hydroxymethyltriazene (Il) was again seen (table 12). Samples of AcMMT decomposed to give three spots on the chromatogram - the arylamine (Xa), the diaryltriazene (VIII) and another spot which is probably the monomethyltriazene itself. The sample of AcMMT as used in this study gave only one peak on HPLC and thus seemed to be a pure compound. Decomposition of AcMMT in phosphate buffer yielded only the amine (Xa) (λ max 312 nm) (figure 58) as described in section D.1.4, with no evidence of formation of the diaryltriazene (VIII) (λ max 380 nm), which is proposed therefore to be the product of a diazomigration reaction (165), catalysed by the slightly acidic silica gel (figure 59). The hydroxymethyltriazene (Il) was always seen as a streak on the plate indicating breakdown during the development of the chromatogram. No discrete spot could be obtained using the tlc system of Julliard *et al* (166), which was employed by these workers to check the purity of the hydroxymethyltriazene (Il).

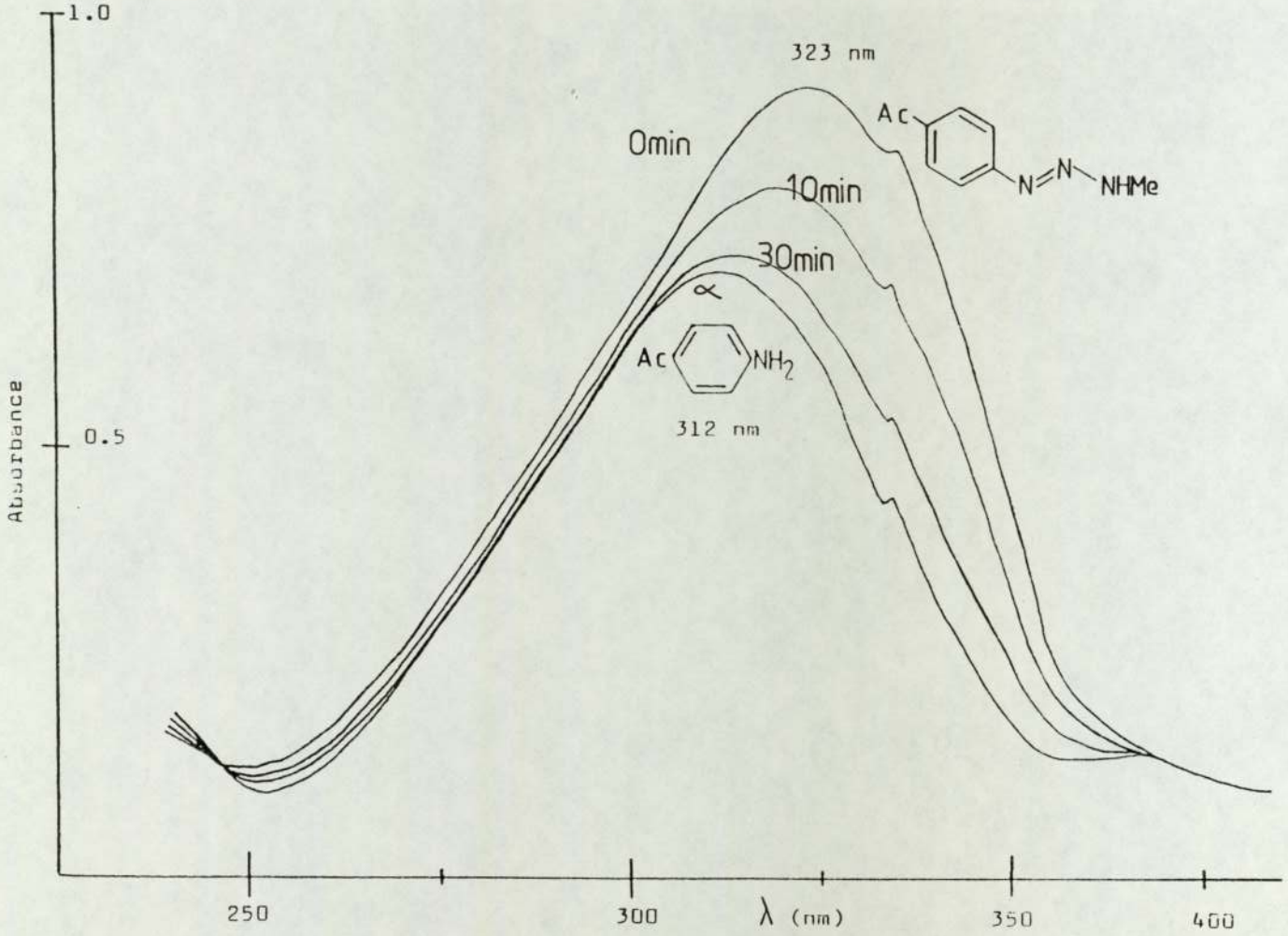


Figure 58 Decomposition of AcMMT in phosphate buffer pH 7.4 in the dark.

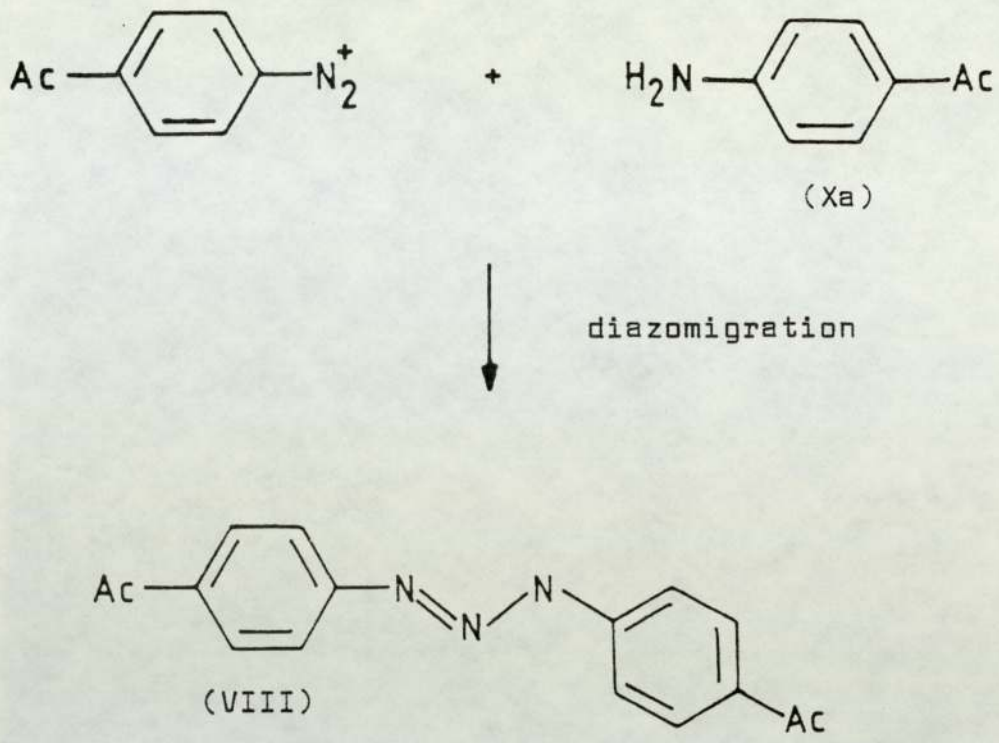


Figure 59 Formation of the diaryltriazene (VIII) by a diazomigration reaction

The tlc system used in the present study would not allow identification of an N-demethylated metabolite of AcDMT other than the arylamine (Xa). However, characterization of the diaryltriazene (VIII), presumably formed as a result of degradation of AcMMT, on in vitro metabolism of AcDMT (table 13) is good evidence for production of the monomethyltriazene (AcMMT). AcMMT is more stable in Earl's buffer than in the phosphate buffer which was used in the present study (table 11), and the former buffer may have been a more suitable medium from which to extract and identify such unstable metabolites of AcDMT. A comparison of the chromatograms obtained using extracts from control and experimental

metabolic incubates of AcMMT, the hydroxymethyltriazene (Il) and the amine (Xa) revealed different patterns of degradation products. This suggests that not only AcDMT, but also proposed intermediates of its metabolism are themselves metabolically degraded by the *in vitro* system.

In vivo, in the rat, 4-aminoacetophenone was seen in urine and plasma samples as a product of metabolism of AcDMT (table 13). Using a more specific and sensitive HPLC method, Farina *et al* identified AcMMT in mouse plasma after i.p. administration of AcDMT (48). The peak plasma level of AcMMT was observed 30 minutes after injection of the parent dimethyl compound, but after 2 hours none was detectable. In the present study, the first blood samples from rats were not taken until 2 hours after dosing with AcDMT, and this probably explains why no AcMMT was detected.

E.5 Metabolism of AcDMT under conditions of the bioassay and comparison with aminopyrine

The metabolism of AcDMT was further investigated in metabolic incubates containing liver fractions and an NADPH generating system (section D.2.2) and comparable to that used routinely in a bioassay in which selective cytotoxicity of dimethyltriazenes (Ix and AcDMT) had been demonstrated (47, 48). The bioassay system is used to activate dimethyltriazenes *in vitro* and to measure the cytotoxic potential of the triazene metabolites (45). The method involves incubation of TLX5 ascites cells with the drug and a liver activating system for 2 hours. After this time, groups of 5 mice are injected i.p. with 0.1 ml of the incubate.

Survival time, which is proportional to the number of viable cells injected, of animals receiving triazene treated cells is compared with that of animals receiving untreated tumour. The advantages of the assay are the ability to control the environment of the tumour cells, for example drug concentration and exposure time, when compared with in vivo studies and a more clear-cut end point compared with in vitro methods.

Since results of the bioassay also showed that a monomethyltriazene (Ix) was equitoxic to triazene sensitive and resistant TLX5 cell lines, it was unlikely that (Ix) was a selectively active triazene species (47). It was suggested that another metabolite, possibly formaldehyde or a formaldehyde precursor, may be of importance in the mechanism of antitumour action (46). Metabolism experiments including aminopyrine, a model substrate of cytochrome P-450 dependent N-demethylation (167, 168), were thought likely to uncover differences between N-methyl compounds and may help to explain why most N-methyl compounds are not cytotoxic although yielding formaldehyde as a product of metabolism.

Metabolism of AcDMT was followed by measurement of substrate disappearance using a normal phase HPLC assay. In closed tubes, metabolism of AcDMT was linear for 30 minutes (figure 12). This is not in agreement with the results of Giraldi (54, 169) and Hill (43), where in vitro triazene metabolism levelled off before this time due either to product inhibition of metabolism (possibly by a diazonium cation or the monomethyltriazene), or to side reactions competing with the demethylation process (54, 169). However, competing side

reactions would not be detected in an assay of substrate disappearance as employed in the present study, and incubation conditions (including access of oxygen) and the identity of the triazene studied in these experiments all differed. Also in the present work, very early assay time points (less than 10 minutes) were not taken, so an initial very fast phase of metabolism could have been missed.

In a bioassay, incubations must be performed in closed tubes to maintain sterile conditions, since the tumour cells are reinjected into mice, and also because continued exposure to oxygen is toxic to TLX5 tumour cells. As expected AcDMT was metabolised to a lesser extent by both mouse and rat 9000g preparations under these conditions than in open beakers (figures 12, 13) since oxygen is a cofactor in the N-demethylation process (170). However, for metabolism to occur at all, it seems unnecessary to flush the flasks with oxygen prior to incubation, a standard practice in other laboratories (45), but not employed in the selective cytotoxicity studies. There is a possibility that triazenes are activated by different mechanisms depending upon the degree of oxygenation, but in further studies on the metabolism of AcDMT in closed tubes (section D.2.3), only products of oxidative metabolism were detected. The carcinogen, dimethylnitrosamine, is metabolised under conditions of partial or total depletion of oxygen (171), and even under a nitrogen atmosphere with production of formaldehyde (172).

No significant differences were found between 9000g and microsomal fractions in the extent of metabolism of AcDMT

in closed tubes, using either rat or mouse liver preparations (table 15). It has been reported that when 9000g rather than microsomal preparations were used to metabolise dimethylnitrosamine, there was a significant increase in formaldehyde liberation and formation of reactive metabolites which covalently bind to nucleic acids (172, 173).

In the present study, a significantly greater quantity of formaldehyde was produced on metabolism of aminopyrine by microsomal rather than 9000g preparations from female CBA/Lac mice (figure 16). Where metabolism of aminopyrine was studied in preparations of mouse or rat liver microsomes made up to volume with microsomal supernatant rather than buffer, a reduced level of species positive in the Nash assay was yielded comparable to that produced on metabolism of aminopyrine by 9000g supernatant (figure 18). This reduction could be caused either by an inhibitor of aminopyrine metabolism in the microsomal supernatant, or by the further metabolism of formaldehyde by the cytosolic enzyme aldehyde dehydrogenase, thus making the formaldehyde unavailable for detection by the Nash assay method. That a difference between the extent of 9000g and microsomal metabolism of aminopyrine still exists when assayed by substrate disappearance (figure 19), [although not significant in another strain of mouse, CBA/Ca (table 19)], suggests that there is a metabolic inhibitor present in the microsomal supernatant. The difference in aminopyrine metabolising capacity between 9000g and microsomal preparations was greater when assayed by the Nash method than by HPLC, possibly due to the effects of

aldehyde dehydrogenase in addition to the activity of a metabolic inhibitor. In contrast, other workers have shown the in vitro metabolism of aminopyrine (0.13mM for 10 minutes) determined by the Nash colorimetric assay to be similar in rat liver 9000g and microsomal preparations (174), whereas less formaldehyde was detected upon N-demethylation of ethylmorphine by rat 9000g when compared with microsomal preparations (174).

AcDMT was easier to extract from metabolism mixtures by ethylacetate (82.8% recovery) than was aminopyrine by dichloromethane (72.4%) (table 14). However, differing extraction efficiencies are unlikely to be the cause of differences between the two metabolic substrates, since the reproducibility of extraction efficiencies was good (table 14). From results of the present study and reports in the literature it thus seems possible that N-demethylation of AcDMT, aminopyrine, dimethylnitrosamine and ethylmorphine occurs by different pathways or is catalysed by different N-demethylase enzymes. There has been a suggestion that there are a number of dimethylnitrosamine demethylases (175), and that demethylation is not a single step mixed function oxidase process (176), but possibly involves an amine oxidase. Both routes of N-demethylation have been documented for N,N-dimethylaniline (figure 60) (177).

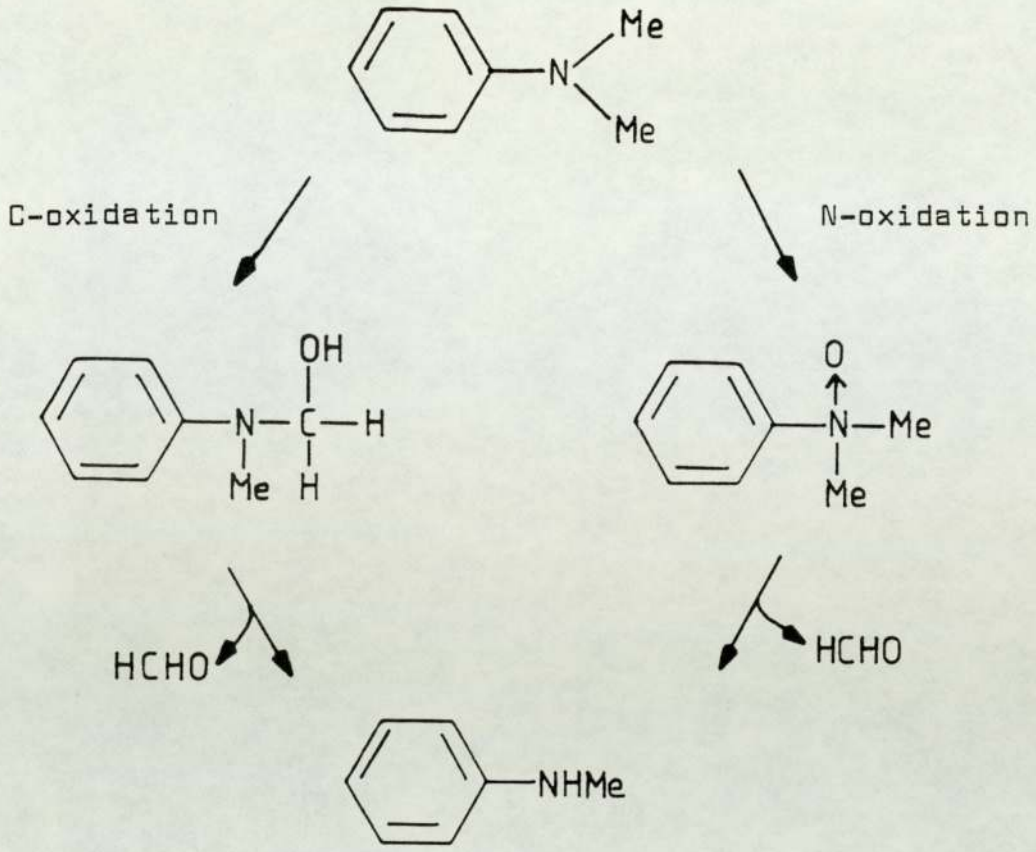


Figure 60 Alternative pathways of N,N-dimethylaniline N-demethylation

A significantly greater extent of microsomal metabolism of aminopyrine (as determined by the Nash assay) was seen in open beakers, but not in closed tubes, using liver preparations from female rather than male mice (table 18). The difference in the extent of metabolism of AcDMT by male and female mouse liver preparations in closed tubes was not significant (table 16). There have been reports previously of sex differences in metabolism primarily in rats (178), but also in some strains of mice (179). N-Demethylation of ethylmorphine and aminopyrine occurs more readily in

microsomes from male rats (180 - 182), whereas with mice a greater degree of metabolism was seen in liver preparations from female animals (179, 181). These results may be explained by an inhibitory effect of androgens on metabolism in the mouse, whereas in the rat, androgens are stimulatory (179).

A similar extent of AcDMT metabolism was seen using either female mouse or female rat microsomal preparations (figure 14). In contrast, a significantly greater degree of aminopyrine metabolism (as determined by either colorimetric or chromatographic assay methods) was observed when using mouse rather than rat liver microsomes (table 17). It is well documented that species differences exist in microsomal monoxygenase activities (183). Differences in aminopyrine demethylase between adult male rat and mouse have been reported to be small (181, 184, 185), but in female animals (as used in the present study), a large species difference has been seen, with the in vitro metabolism of ethylmorphine and aminopyrine 3 - 4 times greater in the mouse than in the rat (181, 186).

In the present work, the extent of metabolism was expressed in terms of the amount of species positive in the Nash assay or the quantity of substrate metabolised per wet weight of liver. There was no difference in the protein content of microsomal preparations from male and female mouse and female rat (table 21), and therefore this can be ruled out as the cause of species and sex differences in metabolism.

In experiments by Connors et al, who employed the bioassay to elucidate the mode of action of antitumour

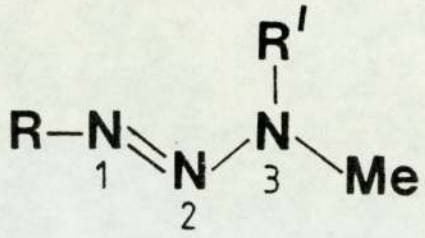
triazenes, 9000g preparations from rats pretreated with phenobarbitone were used (45, 53), whereas in more recent studies in this laboratory, non-induced mouse 9000g liver fractions have been used (47, 48). Metabolism of AcDMT by both microsomal (figure 15) and 9000g fractions in closed tubes was induced when using liver preparations from phenobarbitone pretreated rats. Since AcDMT is metabolised to the same extent by both mouse and rat liver preparations (figure 14), and phenobarbitone pretreatment induces metabolism even under closed conditions (figure 15), it is hard to explain the forcing conditions (flushing with oxygen prior to incubation) required to activate triazenes in bioassays using 9000g preparations from induced rats (45), when other workers using 9000g preparations from non-induced mice were able to activate triazenes incubated in closed tubes (47). An explanation would be that rat and mouse preparations activate triazenes by different mechanisms, but there is no evidence for this.

The inducing effect of phenobarbitone on AcDMT metabolism suggests that under these conditions metabolism of AcDMT is at least partially mediated by cytochrome P-450, since phenobarbitone enzyme induction is specific for the microsomal monoxygenase system (187). Aminopyrine metabolism too was induced by phenobarbitone (table 20). It is probable that only some of the dimethylnitrosamine activation pathways are dependent on cytochrome P-450 (173). Phenobarbitone pretreatment of animals decreased dimethylnitrosame metabolism in rat liver slices to yield carbon dioxide (173), while only slightly dimishing demethylase activity.

There is some evidence that aminopyrine may not be the ideal model substrate of N-demethylation, since other competing metabolic pathways have been proposed (167, 188). Biochemical (189) and analytical problems (190) have become apparent, with neither the Nash colorimetric assay or HPLC determination of metabolites an ideal measure of aminopyrine metabolism. However, the good agreement in the present study between results obtained using the Nash or HPLC assay methods (tables 17, 20) suggests that both methods adequately reflect aminopyrine metabolism. In hindsight, it may be argued that AcDMT was not a good choice of triazene for studies where disappearance of substrate was to be related to N-methyl metabolism since recent studies have shown that the acetyl substituent is also subject to metabolism (section D.2.3).

E.6 Quantification of products formed during metabolism of dialkyltriazenes

The study of the metabolism of AcDMT was extended by determining quantitatively the metabolic products yielded where metabolism was carried out by mouse liver 9000g fraction in closed tubes as employed in the bioassay. Metabolism of other dialkyltriazenes, all at a substrate concentration of 500 $\mu g\ ml^{-1}$ as used in the bioassay studies (47), was also followed by reverse phase HPLC (section D.2.3).

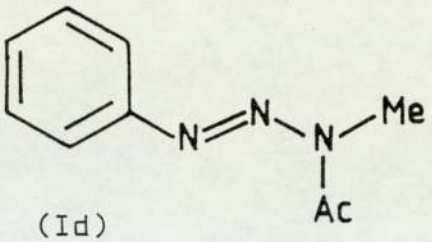


R — carrying structure

R' — preferentially metabolised alkyl group

Figure 61 Structural requirements for triazene antitumour activity.

Structure activity studies of dialkyltriazenes had indicated a requirement for an N-methyl group and another preferentially metabolised alkyl group at N^3 for antitumour activity against TLX5 lymphoma (figure 61) (45, 72). Metabolism of the active antitumour compounds AcDMT and CyDMT yielded the corresponding monomethyltriazenes AcMMT and CyMMT, both with peak concentrations close to 45 $\mu g\ ml^{-1}$ and the arylamines (Xa, Xb) (figures 26, 28). The monomethyltriazene had first been proposed as the active triazene antitumour species in 1969 (41) but not positively identified as a metabolite of a dimethyltriazene before this work. AcMMT has also been shown recently to be an <u>in vivo</u> metabolite of AcDMT (48). Characterization of a 3-acetyl-3-methyltriazene (Id) as an <u>in vitro</u> metabolite of a dimethyltriazene prior to these results had been good evidence for the production of monomethyltriazenes as metabolic intermediates.



(Id)

Diethyltriazenes are not active against TLX5 lymphoma <u>in vivo</u> (45). Metabolism of AcDET and CyDET yielded the inactive monoethyltriazenes AcMET and CyMET (figures 27, 29). 4-Aminobenzonitrile (Xb) was also detected as a metabolite of CyDET; 4-aminoacetophenone (Xa) was presumably formed from AcDET, but, as the sample of dialkyltriazene used here was contaminated with amine (Xa), quantitative analysis was impossible.

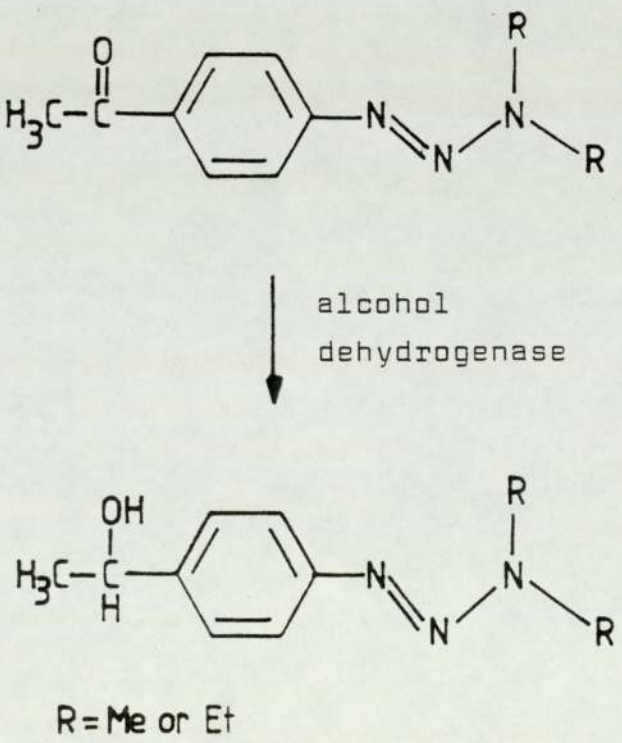
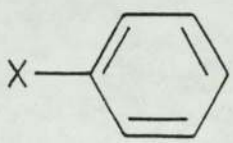


Figure 62 Metabolism of the acetyl substituent in AcDMT and AcDET.

Metabolism of the 4-acetyl substituted triazenes AcDMT and AcDET yielded a more complex pattern of metabolites than the 4-cyano substituted triazenes since the acetyl substituent was reduced to a 1-hydroxyethyl derivative (figures 26, 27, 62). There were no further peaks on the chromatogram which could correspond to dealkylated metabolites of the 4-(1-hydroxyethyl)dialkyltriazenes (Io, Ip), but the polarity of these metabolites is likely to be such that they would not be retained on the column with the HPLC system used. A sample of AcDMT incubated in pH 7.0 sodium phosphate buffer at 24°C with equine liver alcohol dehydrogenase, an enzyme which catalyses conversion between alcohols and aldehydes (or ketones), and NADH, has been shown to yield the same reduced product (Io) (191). In contrast, acetophenone (XVIIa), which also contains an acetyl substituent, was shown to be metabolised by ω-oxidation *in vivo* with formation of benzoic acid (XVIIb), and hippuric acid (XVIIc) (192).



(XVII)

	X	
(XVIIa)	Ac	acetophenone
(XVIIb)	COOH	benzoic acid
(XVIIc)	$CONHCH_2COOH$	hippuric acid

The mean values for the total concentration of products yielded after 2 hours incubation of CyDMT and CyDET with mouse liver 9000g fraction and cofactors were calculated

in terms of equivalents of 4-aminobenzonitrile (Xb). The very similar total concentration of metabolites yielded from CyDMT (equivalent amine (Xb) concentration of 59.9 $\mu g\ ml^{-1}$) and CyDET (equivalent amine (Xb) concentration of 58.0 $\mu g\ ml^{-1}$) (figures 28, 29) suggests that both substrates undergo metabolism to yield the monoalkyltriazene and amine to the same extent. In contrast, the extent of dealkylation of the diethyl analogue of aminopyrine (XIe) by liver homogenates has been reported to be half that of dealkylation of aminopyrine itself (167). Chau and co-workers also demonstrated a greater Vmax for formaldehyde release from dimethylnitrosamine than for acetaldehyde from diethylnitrosamine (193).

The 4-cyanophenylmethylethyltriazene (Iu) yielded both CyMMT and CyMET on metabolism (figure 63), with CyMMT reaching a greater peak concentration than CyMET, and also 4-aminobenzonitrile (Xb) (figure 30). Methylethyltriazenes are active antitumour compounds against TLX5 lymphoma, and it has been proposed that N-dealkylation would occur preferentially in the longer alkyl chain affording the active monomethyltriazene (45). Chau et al, working on the oxidative dealkylation of methylethylnitrosamine, found that the Vmax for acetaldehyde release was nearly twice that for formaldehyde release, indicating that this compound is preferentially de-ethylated (193).

In the present study, it is difficult to be sure whether the higher yield of the monomethyltriazene (CyMMT) when compared with the monoethyltriazene (CyMET) produced on metabolism of the methylethyltriazene (Iu) is due to

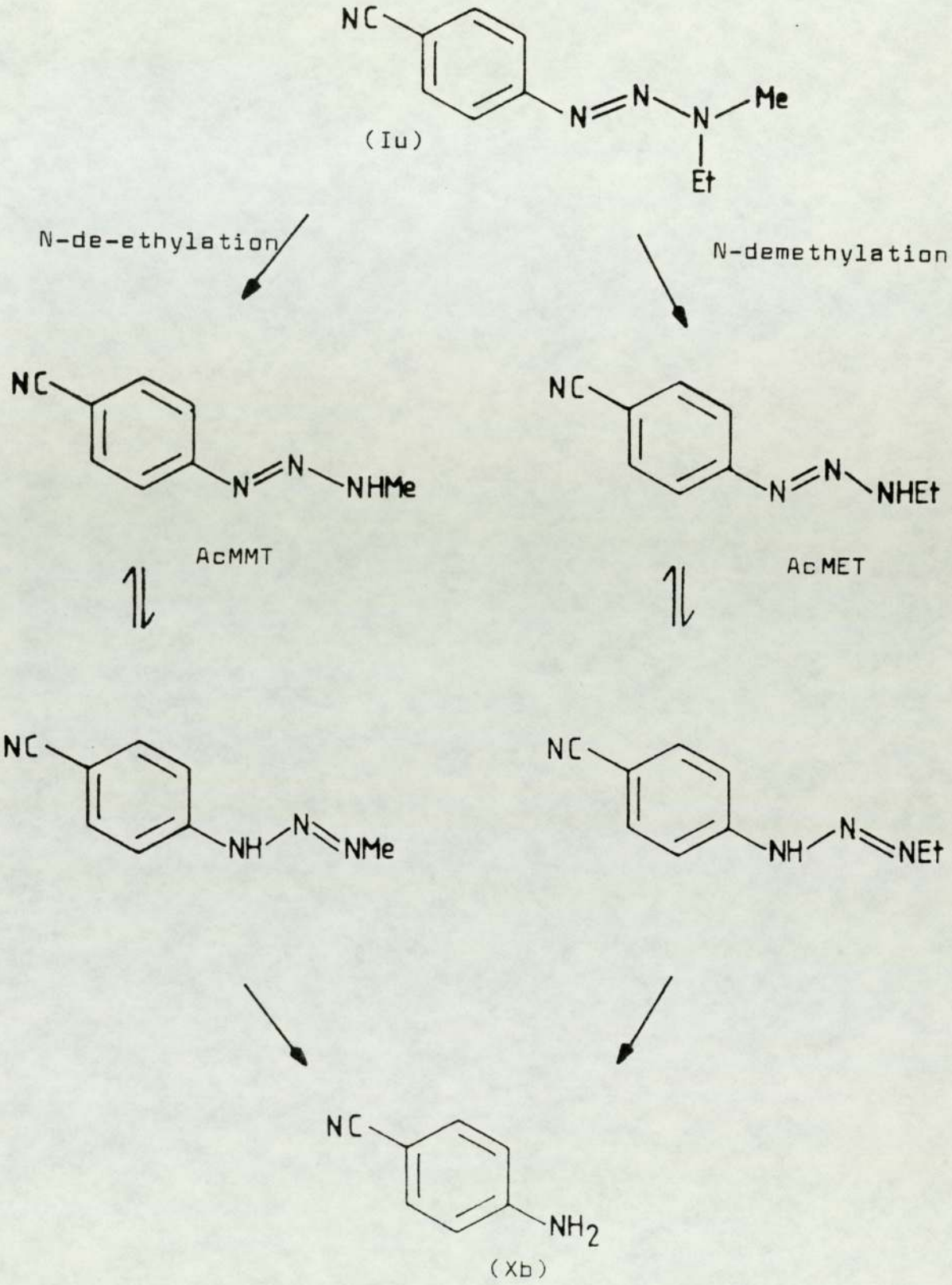


Figure 63 Alternative N-dealkylation pathways for 1-(4-cyanophenyl)-3-methyl-3-ethyltriazene (Iu)

preferential de-ethylation of the substrate, or results from the comparatively short half-life of CyMET (table 22) so that it decomposes more quickly than CyMMT once formed. The production of monoalkyltriazenes from 1-(4-cyanophenyl)-3-methyl-3-ethyltriazene (Iu) followed a time course that would be predicted from the production of metabolites from CyDMT and CyDET, although for (Iu) peak levels of metabolites were lower. Since a greater concentration of 4-aminobenzonitrile (Xb) was produced on metabolism of CyDET than CyDMT, although total metabolite concentrations were the same, it may be presumed that the low concentration of CyMET detected on metabolism of CyDET (figure 29) is due to its rapid decomposition, once formed, to the amine (Xb), rather than to a low metabolic yield. Since the stability of CyMET will be the same from whatever substrate it is afforded under the same incubation conditions, it is likely that, in the case of the methylethyltriazene (Iu), significant demethylation as well as de-ethylation occurs.

The only major product of metabolism of the methyl-tert-butyltriazene (Iv) was 4-aminobenzonitrile (Xb) (figure 31). This suggests that demethylation of (Iv) occurred to yield the presumably very unstable mono-tert-butyltriazene (Iw), possibly seen as an extremely small peak on the chromatogram after 15 minutes incubation. This monoalkyltriazene (Iw) would decompose quantitatively to yield the arylamine (Xb). The methyl-tert-butyltriazenes have been proposed to be inactive since no monomethyltriazene may be produced on their metabolism, as the tert-butyl group has no α - CH bond and cannot be dealkylated (figure 64). The present result is in agreement with this hypothesis.

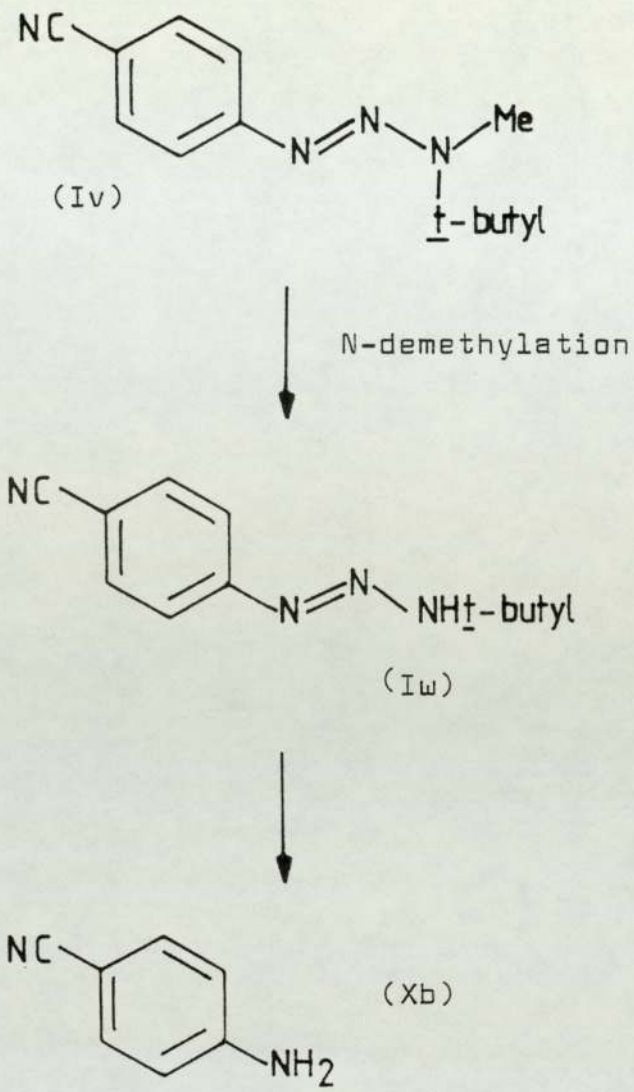


Figure 64 Metabolic pathway for 1-(4-cyanophenyl)-3-methyl-3-t-butyltriazene (Iv)

The peak concentration of AcMMT (49 $\mu g\ ml^{-1}$) yielded upon metabolism of AcDMT (figure 26), is greater than that reported to be afforded from AcDMT in a bioassay ($< 2\ \mu g\ ml^{-1}$) (48). It is close to the level of 60 $\mu g\ ml^{-1}$ AcMMT proposed to be required to elicit cytotoxicity to TLX5R cells equivalent to that produced on activation of 500 $\mu g\ ml^{-1}$

AcDMT (48), and suggests that AcMMT could be responsible for the cytotoxic effects of AcDMT in the bioassay.

No unknown HPLC peaks were observed in the study, and it appears that, under these conditions, the major pathway of metabolism, if not the sole pathway, is N-demethylation, unless products of other metabolic pathways are yielded but not detected by the HPLC assay. This is puzzling considering the range of metabolites characterized by Pool (69) on *in vitro* metabolism of 1-phenyl-3,3-dimethyltriazene (Ia). 1-Aryl-3,3-dimethyltriazenes are not activated by TLX5 cells (47), and it is unlikely that the tumour cells can metabolise the triazenes by other pathways not resulting in activation. A monomethyltriazene, AcMMT, has also been shown not to be metabolised by TLX5 tumour cells (section D.2.5). It is therefore probable that metabolism of dimethyltriazenes in the closed tubes is by the same pathway regardless of whether TLX5 tumour cells are present. The results suggest that a demethylated product of a dimethyltriazene is responsible for its selective cytotoxicity in the bioassay (47), and yet there is evidence against the involvement of both the monomethyl- and hydroxymethyl- triazene in the rôle of selectively active species (47, 51).

E.7 Metabolism of monoalkyltriazenes and arylamines

One of the major criticisms of the theory that the monomethyltriazenes are the active triazene antitumour species is the finding that the diethyltriazenes are not antitumour agents although they are de-ethylated (53), with the resulting monoethyltriazenes being just as potent alkylating agents *in vitro* as the monomethyltriazenes (92). It is hard to explain

why monoethyltriazenes metabolically formed *in vivo* are not as cytotoxic as the corresponding monomethyltriazenes produced in the same way, if the reason for cytotoxicity is an alkylating mechanism. A possible explanation is that monoethyltriazenes are more rapidly deactivated by chemical and metabolic degradation than the corresponding monomethyltriazenes once formed *in vivo*, and are therefore not available as alkylating species. Experiments were therefore undertaken to compare the susceptibility of monoalkyltriazenes to degradation by metabolism (section D.2.4).

The three monomethyltriazenes (AcMMT, CyMMT, Idd) were shown to be metabolised in an incubation system containing mouse liver 9000g fraction and cofactors (figures 32 - 34, 37 - 39). Metabolism of the monoethyltriazenes AcMET and CyMET was also demonstrated (figures 37, 38, 40). Disappearance of substrate was determined by reverse phase HPLC, and metabolism was seen as the increased disappearance of the triazenes in experimental incubates when compared with control incubates of the same composition, but containing liver inactivated by heating.

It is not clear whether metabolism of these monoalkyltriazenes occurs at the N-alkyl moiety because products other than the arylamines were not identified. For two of the monomethyltriazenes studied (AcMMT and Idd), metabolism of the aryl ring substituent could occur. Nitro groups are known to be reduced (194), and metabolism of the acetyl substituent was demonstrated in section D.2.3. The

metabolic rate for CyMMT presumably represents metabolism of the monomethyl moiety, unless ring hydroxylation occurs, but this is thought to be only a minor pathway in *para*-substituted triazenes (57).

The two arylamines (Xa, Xb) were also metabolised under the same conditions employed for monoalkyltriazene metabolism (figures 35, 37). 4-Aminoacetophenone (Xa) was metabolically decomposed at a faster rate than 4-aminobenzonitrile (Xb), probably due to the metabolism of the acetyl group in addition to metabolism of the amine. Both arylamines were stable in control incubates.

The products of metabolism of the arylamines are unknown, no extra peaks were seen on the chromatogram. Aromatic amines are known to undergo hydroxylation to yield hydroxylamines (195, 196) (figure 65), and the hydroxylase is a microsomal enzyme requiring oxygen and NADPH just as the demethylase enzyme. Aniline itself is not readily N-hydroxylated, but *para*-substituted amines (for example *p*-chloroaniline) are much more readily metabolised by this pathway (195). A minor metabolic route for amines is by acetylation (197) (figure 65), and the N-acetyl derivative of 4-aminoantipyrine (XIg) has been detected after administration of aminopyrine to rat and man (198). *In vitro*, 4-aminoantipyrine (XIc) has been shown to be metabolised to a small extent by whole liver, but is stable in the presence of microsomes (167).

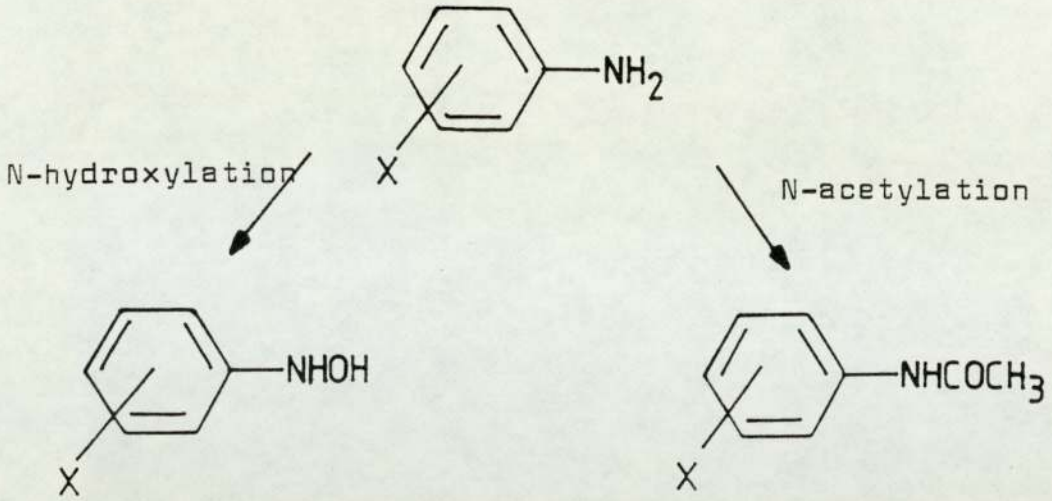


Figure 65 Possible metabolic pathways for aromatic amines

The half-lives of the monoalkyltriazenes in metabolism and control incubates (table 22) were determined from measurements of substrate disappearance in the high concentration incubates (50 and 100 $\mu g\ ml^{-1}$) where there was still substrate remaining after 30 minutes. The monoethyltriazenes were found to be less stable than the corresponding monomethyltriazenes under each of the different incubation conditions. The more stable the triazene, the more available it is for metabolism, and the greater the difference between half-lives determined in control and experimental incubates (table 22).

At a concentration of 100 $\mu g\ ml^{-1}$, the metabolic half-lives as a percentage of the control half-lives were 53.1% and 49.1% for AcMMT and AcMET, and 66.0% and 75.2% for CyMMT and CyMET respectively. The results do not show a more rapid metabolism of monoethyltriazenes than the corresponding monomethyltriazenes. This cannot therefore be the reason for the inactivity of monoethyltriazenes in vivo,

unless *in vivo* metabolism is very different from that in the *in vitro* system used here. Although the monoethyltriazenes used in the present study (AcMET, $t_{\frac{1}{2}}$ 15.1 min: CyMET, $t_{\frac{1}{2}}$ 18.3 min) are more chemically unstable in Earl's buffer pH 7.4 at 37°C than the corresponding monomethyltriazenes (AcMMT, $t_{\frac{1}{2}}$ 66.6 min: CyMMT, $t_{\frac{1}{2}}$ 99.0 min) (table 22), they are not as labile as the trichloromonomethyltriazene (Ibb) for example ($t_{\frac{1}{2}}$ at 37°C, 1.2 min in pH 7.4 phosphate buffer) (table 11), which is thought to be afforded *in vivo* from the active dimethyltriazene (Iaa) (50).

The half-lives of three out of the four monoalkyltriazenes studied were greater in Earl's buffer pH 7.4 (chemical $t_{\frac{1}{2}}$) than in control incubations in the presence of heat-inactivated liver (control $t_{\frac{1}{2}}$). The difference was most apparent for the monomethyltriazenes AcMMT (control $t_{\frac{1}{2}}$, 33.4 min: chemical $t_{\frac{1}{2}}$, 66.6 min) and CyMMT (control $t_{\frac{1}{2}}$, 59.6 min: chemical $t_{\frac{1}{2}}$, 99.0 min). Possibly heating of the liver exposes some nucleophilic site which catalyses the decomposition of the monoalkyltriazenes. The result is in contrast with the observations of Delben *et al*, where protein in solution interacted with phenylmonomethyltriazenes increasing their stability (161). An alternative explanation is that the solution of cofactors added with the heat-inactivated liver to control incubates makes conditions slightly more acidic, thus increasing triazene decomposition rates.

In control incubates, 91.8-105.0% of the monoalkyltriazenes decomposed over 30 minutes could be accounted for

by the appearance of arylamine (table 23). However, in experimental incubates not as much arylamine was detected as the theoretical yield calculated from the quantity of triazene decomposed, particularly in the case of the monomethyltriazenes AcMMT (70.4% of the theoretical yield detected) and CyMMT (58.3%) (table 23). This could be due partly to further metabolism of the amine itself, but for 4-aminobenzonitrile (Xb), amine metabolism has been shown to be of minor importance. The results presented in table 23 suggest that the actual yield of arylamine detected by HPLC in the experimental incubates is more closely related to the yield expected from the quantity of monomethyl-triazene decomposed by chemical degradation, rather than the total quantity decomposed by both chemical and metabolic degradation. Metabolism of monoalkyltriazenes may therefore yield a product other than the corresponding arylamine. The difference between theoretical and actual yield of arylamine in experimental incubates was no greater for the acetyl-substituted triazenes than for the cyano-substituted triazenes, suggesting that little if any of the acetyl substituent was reduced as occurs during the metabolism of AcDMT (section D.2.3).

La Du reported that upon metabolism of aminopyrine or 4-methylaminoantipyrine (XIb), about twice as much substrate disappeared <u>in vitro</u> than could be accounted for by the appearance of formaldehyde and amine (167). Recently, 4-formylaminoantipyrine (XId) has been detected as a metabolite of both aminopyrine and its monomethyl analogue

in vitro and in vivo (188, 190), and this could explain the discrepancy. A similar metabolic pathway is conceivable in the case of the monomethyltriazenes (figure 66). The ∝C-hydroxylation of the monomethyltriazene to yield a stable monohydroxymethyl intermediate (Iee) would also explain the low levels of arylamine (table 23) and formaldehyde (45, 54, 58) detected during the metabolic process (figure 66). As yet this type of compound (Iee) has not been synthetically accessible and its stability is unknown.

N-Methyl-4-aminoazobenzene (MAB) is oxidised to N-hydroxy MAB by rat liver microsomes (196). This is proposed to be an activating step, with the N-hydroxy metabolite a proximate carcinogen. If the monomethyl- and monoethyl- triazenes are metabolised by different pathways, but only for the monomethyltriazenes is metabolism an activating process, this could possibly explain the lack of in vivo antitumour activity of the monoethyltriazenes. However, there is some preliminary evidence suggesting that in vitro the metabolism of a monomethyltriazene results in deactivation (45, 47, 199). In one experiment, AcMMT at a concentration of 50 $\mu g\ ml^{-1}$ was incubated with TLX5 cells for 2 hours under conditions of the bioassay either alone, in the presence of a full metabolising system, or with liver or cofactors only. The percent increase in life span (ILS) of mice injected with cells from the different incubates is shown in table 30. The decreased percent ILS in the presence of the full metabolising system may be due to a deactivation

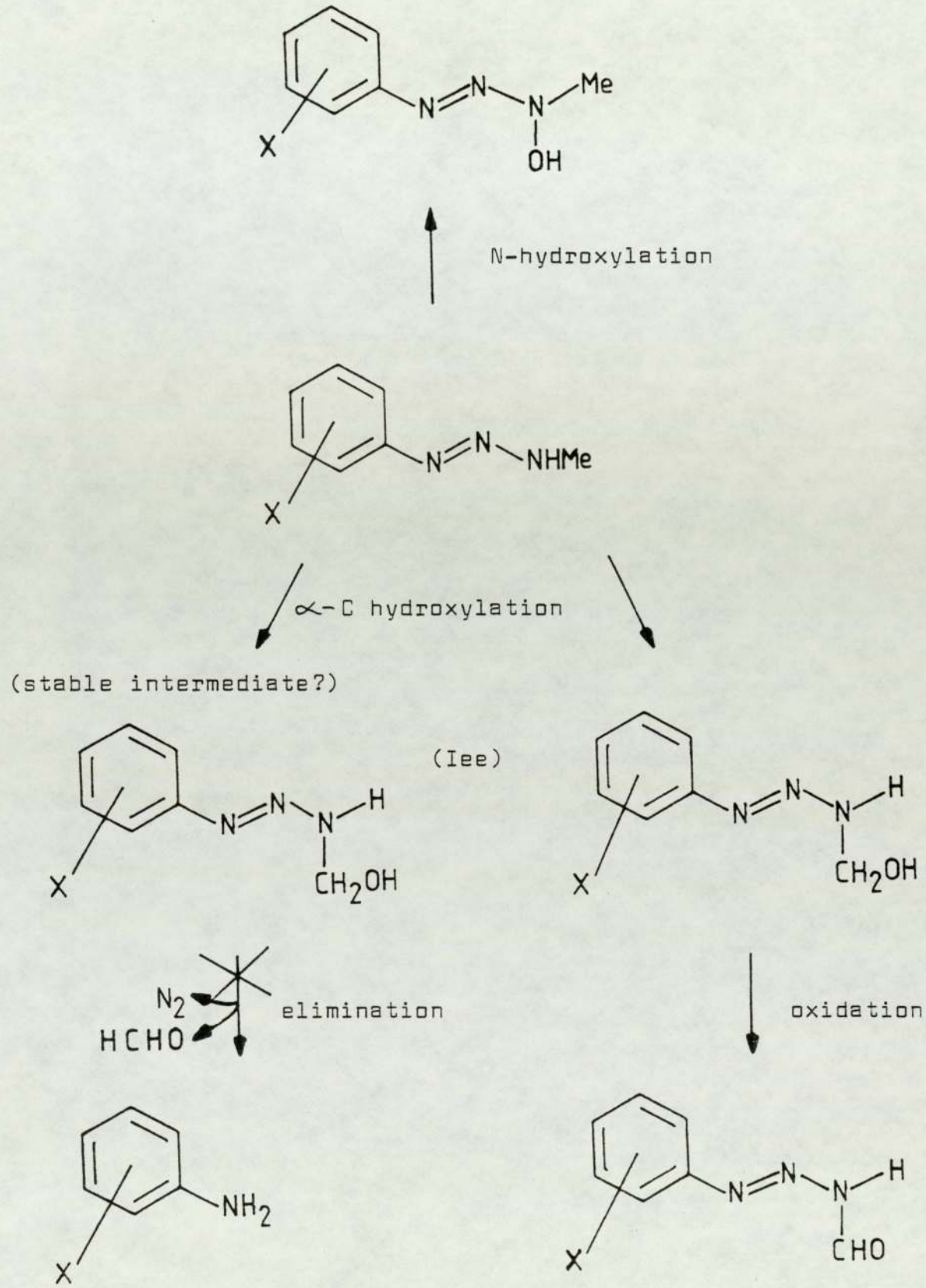


Figure 66 Possible metabolic pathways for an arylmonomethyltriazene

of AcMMT by metabolism. However, as the percent ILS is partly reduced where cells were incubated with AcMMT and cofactors only, it is probable that the acidic nature of the cofactors is to some extent responsible for AcMMT deactivation by causing chemical decomposition of the AcMMT to the inactive arylamine (Xa). HPLC assay of the AcMMT levels in each of the incubates, including a control incubate containing cofactors and liver inactivated by heating would help clarify the result.

Table 30 Bioassay of AcMMT under different incubation conditions.

Incubation conditions	Percent ILS*
AcMMT alone	51
+ 9000g liver homogenate	72
+ cofactors	32
+ liver and cofactors	9

* 60% ILS $\geqslant$ 5 log cell kill

E.8 Toxicity of AcDMT and AcMMT to mouse hepatocytes

The search for the identify of the active metabolite derived from a dimethyltriazene, AcDMT, was continued in a mouse hepatocyte system (section D.3.1). The hepatocyte is a good model system since the triazene may be metabolised

(either activated or deactivated) and the combined toxicity of substrate and metabolites determined *in situ*. The toxicity of AcDMT and AcMMT was determined by the trypan blue dye exclusion test, which, in separate experiments has been shown to give good agreement with an assay for leakage of lactate dehydrogenase (LDH) - a marker enzyme for cell membrane integrity.

After incubation of hepatocytes for one hour with AcDMT or AcMMT, no decrease in viability of the cells was seen in comparison with control hepatocytes, except at the highest concentration of AcDMT employed, 1.5mM (31% increase in trypan blue staining in experimental compared with control incubates). The relatively low toxicity of the triazenes could be due to either low intracellular levels of the toxic species because of poor uptake or deactivation by further metabolism. Alternatively, any event leading to cytotoxicity may not be apparent immediately as a decrease in viability as determined by the dye exclusion assay.

After 2 hours incubation, AcMMT was significantly less toxic to the hepatocytes than AcDMT (figure 42). Dimethyltriazenes are not cytotoxic to TLX5 tumour cells until metabolically activated (47). Similarly in the present study, it is probable that AcDMT was non-toxic to hepatocytes before it had been metabolised. It is unlikely that the total amount of AcDMT in any incubate would be activated by metabolism and hence the difference in cytotoxicity between equimolar concentrations of AcMMT and activated AcDMT is even greater than the difference between AcMMT and AcDMT apparent in this study.

A possible explanation for these results is that AcMMT is not responsible for the decrease in cell viability produced on incubation of hepatocytes with AcDMT. The activated species yielded from AcDMT appears to be more toxic than AcMMT, although the toxicity of a compound added externally and then incubated with the cells may be very different to the toxicity of the same compound metabolically produced at a constant rate within the cells. The lower level of toxicity to the hepatocytes when incubated with AcMMT may be due to the fact that AcMMT is metabolically deactivated as it is taken up into the hepatocyte. Such a rapid deactivation step for AcMMT also suggests that AcMMT is not responsible for the decrease in cell viability caused by AcDMT, since AcMMT generated within the cells from AcDMT (48) should be deactivated as rapidly as the AcMMT taken up into the hepatocytes.

In all experiments of this type comparing two agents, results are valid only if both compounds are taken up into the cells at a similar rate. This may not be so if lipophilicities differ greatly, or if uptake is prevented by protein binding within the incubation medium. Results may also be affected by the instability of AcMMT compared with AcDMT. The stability of AcMMT was not determined in the Kreb's incubation medium used in the present study, but at $37^{o}C$ in Earl's buffer at pH 7.4 in the presence of liver it had a half-life of 33.42 minutes, whereas AcDMT was stable.

Connors et al (45) similarly compared cytotoxicity of a dimethyl- and a monomethyl- triazene (Ih, and Ig), but

against TLX5 lymphoma cells with and without a microsomal metabolising system. Their results, like those in the present study, suggest that, for monomethyltriazenes, metabolism is a deactivating step. Assuming that the monomethyltriazene is responsible for the cytotoxicity of the dimethyltriazene, it is difficult to explain why the monomethyltriazene produced on activation of the dimethyltriazene is not immediately deactivated by the same system. The study by Connors et al using tumour cells is different from the study in hepatocytes presented here however, since the monomethyltriazene could be taken up into the TLX5 tumour cells where there is no further metabolism (section D.2.5), whereas in hepatocytes, the triazene may be further metabolised within the target cell.

It is not known if the cytotoxicity measured in this study in hepatocytes is produced by the same mechanism as that resulting in the antitumour activity of the triazenes to TLX5 cells. It seems probable that it is non-specific cell death that is observed, comparable to the cytotoxicity to TLX5R cells, rather than the selective action reported to occur in the TLX5 sensitive line (47).

E.9 Toxicity of AcDMT and AcMMT to TLX5 lymphoma ascites cells

Some doubt has been cast on the contention that monomethyltriazenes are the species responsible for the selective cytotoxicity of dimethyltriazenes, since, in a bioassay, a monomethyltriazene (Iy) was equitoxic to both triazene sensitive and resistant TLX5 cell lines, whereas the parent dimethyltriazene (Ix) was, on activation, more

cytotoxic to the sensitive line (47). The results obtained in the present study using another monomethyltriazene, AcMMT, and determining cytotoxicity to TLX5 cells (decrease in cell viability) by the dye exclusion test were not in agreement with the bioassay results. The significantly decreased cell viability of the sensitive line incubated with AcMMT when compared with the resistant line (figure 43), now suggests that the monomethyltriazene may be responsible for the selective cytotoxicity of a dimethyltriazene.

It is possible that the cytotoxicity measured in the present work is not equivalent to that measured in the bioassay. Lower concentrations of AcMMT ($<100\ \mu g\ ml^{-1}$) which produced an increase in life span in animals in a bioassay (48), were not cytotoxic as determined by the dye exclusion test, although incubation with the triazene lasted for 2 hours in each study. In a bioassay, it is not necessarily immediate cell death that is being measured, as in the present work. It may be that the triazene is able to inhibit further cell growth or replication, or the utilization of some factor required for the functioning of the cell - events that result in cell death at a later time. Alternatively, the bioassay may be an artifactual system in which true cytotoxicity is not measured. The triazene may exert its effect by altering the immunogenicity of cells (119) leading to tumour rejection by the animals and an increase in life span in the bioassay, a mechanism that would not be apparent in the results of an <u>in vitro</u> dye exclusion assay. The

results obtained in the present study are, however, in agreement with those of an *in vivo* study of a monomethyltriazene (Iy) against TLX5 lymphoma, where preferential cytotoxicity was seen to the triazene sensitive line, and lends support to the proposal that it is the bioassay results that are misleading.

The fact that incubation of AcDMT with TLX5 cells caused no additional decrease in cell viability when compared with control untreated cells, is further evidence that the tumour cells cannot activate dimethyltriazenes. This is in contrast to work with hepatocytes (section D.3.1) where AcDMT was activated and is cytotoxic.

E.10 Mechanism of triazene resistance in TLX5 lymphoma

The mechanism of development of resistance to the antitumour activity of activated dimethyltriazenes in TLX5 cells is unknown. In general, resistance is thought to occur as a result of differences between the resistant and sensitive cells in any of the biochemical factors required for drug action. As dimethyltriazenes must be metabolically activated to yield antitumour species, it is possible there exists a difference in the metabolising capacity of the two TLX5 cell lines. It has been shown in the present work that neither the TLX5 line sensitive or the one resistant to triazenes is able to activate a dimethyltriazene (section D.3.2), or to metabolise a monomethyltriazene, AcMMT, (possibly the active antitumour species) as determined by HPLC (figure 4).

An alternative mechanism of resistance, possibly resulting in a difference in the concentration of the active

species at the site of antitumour action, is a difference in the cellular concentration of protective agents such as sulphydryl compounds. Glutathione levels were assayed in both triazene sensitive and resistant TLX5 cell lines (section D.4.1), bearing in mind the glutathione depleting effects of AcMMT (section D.4.2). Total glutathione levels (presumably mostly GSH since GSSG levels were below the detection limit) were the same in both TLX5 lines (table 24), and therefore this is presumably not the mechanism of triazene resistance. In an L1210 cell line made resistant to L-phenylalanine mustard (L-PAM), GSH levels were reported to be 2-4 fold higher than in the sensitive line (200) and a reduction of glutathione levels in the sensitive line sensitized it to the antitumour activity of L-PAM.

E.11 Influence of triazenes on glutathione levels in mouse liver preparations

The presence of reduced glutathione (the major non-protein thiol in body tissues) is essential for the normal functioning of cells (201). Hepatic concentrations in mice are particularly high when compared with the levels in other organs (202). In the liver, glutathione serves many rôles such as intracellular reductant, detoxificant and cofactor. Functions of glutathione have recently been reviewed (203), and more specifically its relationship to drug metabolism described by Orrenius and Jones (204).

The following observations were made in the present study concerning the interaction between triazenes and this important tripeptide:

i) A dimethyltriazene (AcDMT) and to a minor extent a monomethyltriazene (AcMMT) were found to interfere with the assay of standard solutions of GSH, resulting in a reduction of the assay values obtained. This was a problem for AcDMT when measuring total glutathione levels in mouse liver homogenate, but AcMMT did not interfere with the glutathione assay under the conditions of liver incubates.

ii) AcMMT and a series of other monoalkyltriazenes were able to prevent the oxidation of GSH to GSSG in mouse liver homogenates incubated in Earl's buffer, whereas their decomposition products, the arylamines, could not prevent GSH oxidation.

iii) AcMMT was shown to deplete total glutathione levels in mouse hepatocytes, and the increase in extracellular glutathione concentration could not account for the intracellular loss.

iv) Neither AcDMT or AcMMT were able to inhibit the activity of glutathione reductase in mouse hepatocytes, whereas BCNU was a potent inhibitor under the same conditions.

In the following sections, these triazene effects are discussed in more detail.

E.11.1 Interference by triazenes with the assay of standard GSH solutions

When the interaction between triazenes and GSH was studied, a decrease in the analytical reading for a standard amount of GSH was observed in the mere presence of AcMMT and particularly AcDMT (table 25). This could be due either to the inhibition of glutathione reductase in the assay by the

triazenes, or to a chemical interaction between triazene and glutathione thus removing free GSH which was determined in the assay. Further studies showed that the triazenes do not inhibit glutathione reductase (section D.4.3). Without doubt diazonium species are formed from the triazenes under acidic conditions such as in 10% metphosphoric acid (126), conditions applied in the assay because GSH is least susceptible to oxidation in acidic media. These diazonium species might couple with the thiol group of glutathione as described previously for cysteine in section D.1.2.1. (figure 67a). For AcDMT, this is the only conceivable mechanism of chemical interaction with glutathione; AcMMT is presumably also able to methylate GSH resulting in the formation of S-methylglutathione (figure 67b).

The free glutathione remaining in solution in the presence of AcDMT was all in the oxidised form. AcMMT was similarly able to cause oxidation of glutathione, and in this case the degree of oxidation was dependent on the concentration of AcMMT (figure 38).

When using the glutathione assay to determine glutathione levels in mouse liver homogenate (section D.4.4), AcMMT at concentrations of 1mM or below was found not to interfere with either the total glutathione assay reading, or the degree of GSH oxidation in the liver preparation. However, the addition of AcDMT to liver homogenate preparations resulted in a decrease in the total glutathione assay value to 25% of the level without triazene. Of the remaining free glutathione, 35% was in the oxidised form, as compared

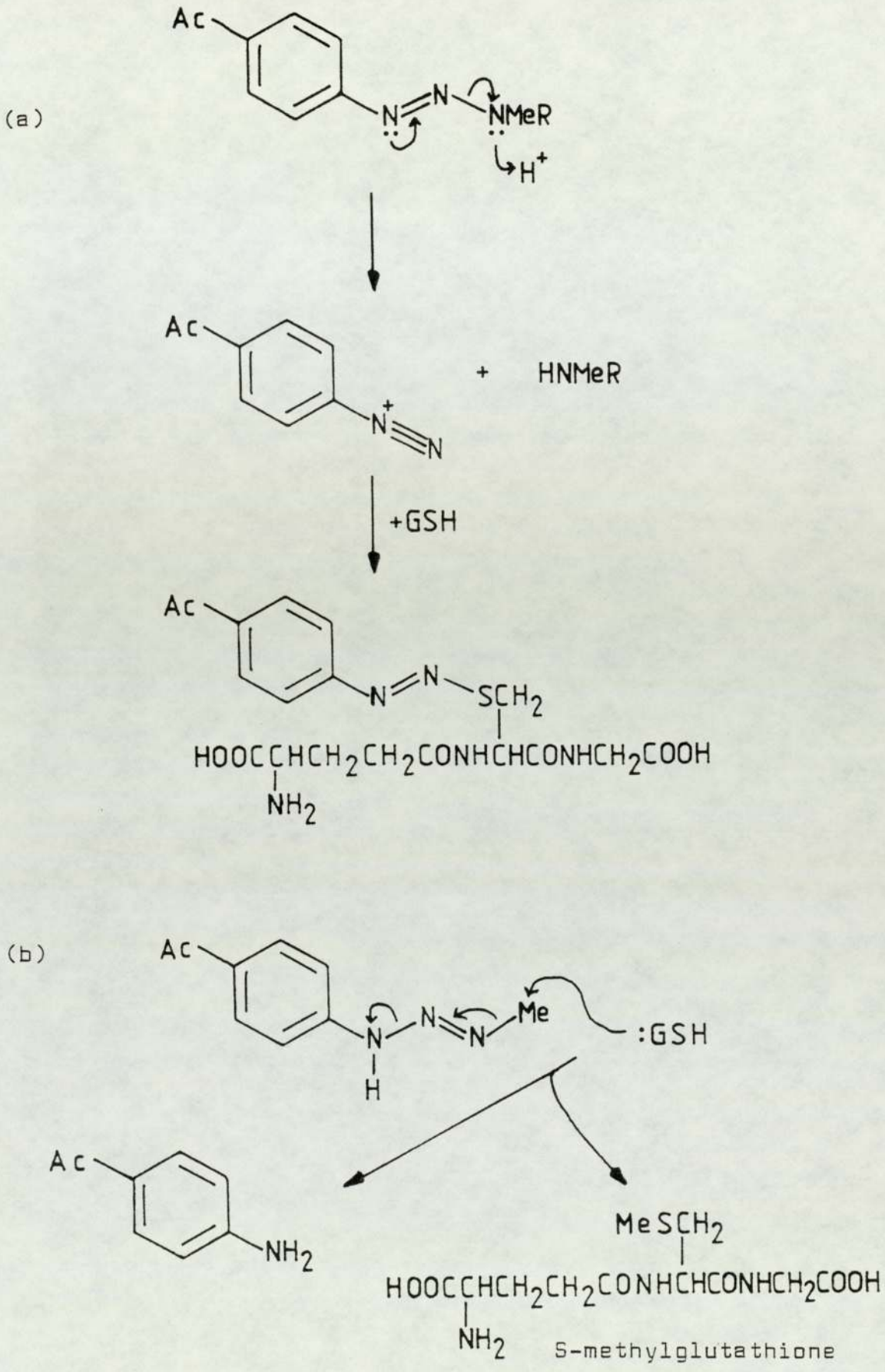


Figure 67 Proposed mechanisms of chemical interaction between triazenes and glutathione a) by coupling b) by methylation.

with 3% in control homogenates, or in homogenates in the presence of AcMMT. The glutathione assay method was therefore considered to be applicable to study the interaction of AcMMT, but not AcDMT, with glutathione in mouse liver homogenate and mouse hepatocytes.

E.11.2 The effect of triazenes on glutathione oxidation in mouse liver homogenate

Rapid oxidation of GSH to GSSG was found to occur in mouse liver homogenate incubated at 37°C in Earl's buffer pH 7.4, with formation of 100% GSSG in 30 minutes (fugure 48). GSH in mouse kidney homogenate incubated in sodium phosphate buffer has been shown to be much more prone to oxidation than GSH in mouse liver homogenate (205). Conversion of GSH to GSSG has been observed in suspensions of kidney cells (206), in plasma (207) and to a lesser extent in medium without cells (208), but not in 0.2M phosphate buffer pH 7.0 (209).

In kidney preparations, oxidation has been proposed to be due to the action of γ-glutamyl transpeptidase on GSH, with subsequent non-enzymatic oxidation and transhydrogenation reactions of the yielded cysteinylglycine resulting in formation of GSSG (210). The tissue distribution of the glutathione oxidase and the γ-glutamyl transpeptidase, a membrane bound enzyme abundant in kidney but not in liver (211), are similar (212), and an oxidation mechanism involving γ-glutamyl transpeptidase thus explains the low levels of oxidation in the liver. Further purification procedures have resulted in isolation of a glutathione oxidase with no γ-glutamyl transpeptidase activity (213). In more recent studies, rapid autoxidation of glutathione has been seen in extracts from

perfused rat liver, even after freeze clamping and perchloric acid deproteinisation (214). It is therefore probable that oxidation of GSH can take place in tissue preparations without the involvement of γ-glutamyl transpeptidase.

In the present study, addition of monoalkyltriazenes AcMMT, AcMET, (Ig) and (Iy) to the mouse liver homogenate incubation at a concentration of 1mM resulted in a reduction of the glutathione oxidation rate (figure 50, table 28). Two arylamines (Xa, Xc) and another monomethyltriazene, CyMMT, also incubated at a concentration of 1mM, were, however, not able to influence the rate of glutathione oxidation (figures 50, 51). In an attempt to elucidate the mechanism of this oxidation inhibition by some triazene derivatives, the nature of glutathione oxidation in liver homogenate was further investigated.

The glutathione oxidation in the liver homogenate appeared to be catalysed by metal ions, since EDTA was partly able to inhibit oxidation, although only at very high concentrations (10, 15mM) (table 29). In rat kidney plasma membrane fraction, 1mM EDTA has been shown to cause 84% inhibition of oxidation (215), whereas in the liver preparation used in the present work, the same concentration of EDTA had only a minor effect. Also, contrary to results obtained for the kidney (isolated renal cell preparations) (213), the copper chelator diethyldithiocarbamic acid had no inhibitory effect on oxidation rate. In the case of plasma GSH, addition of EDTA had no effect on its disappearance rate (209), and in perfused rat liver, glutathione oxidation was only partly suppressed by EDTA (214).

Metal ions may be present in the Earl's buffer used in the incubation, or in the liver homogenate. The rate of glutathione oxidation in a preparation of liver perfused with sucrose prior to homogenisation was the same as in non-perfused liver preparations suggesting that contamination with blood was not responsible for the oxidation of GSH (figure 49). Some autoxidation of GSH was seen in a solution of GSH made up in Earl's buffer and incubated at $37^{o}C$, although at a rate significantly lower than the rate of oxidation of glutathione in liver homogenate (table 27). A lesser oxidation rate was seen where the homogenate was incubated with sucrose rather than Earl's buffer (figure 48), suggesting that buffer composition does influence glutathione oxidation.

The lack of oxidation of glutathione on incubation of the liver homogenate under nitrogen (figure 48) suggests that molecular oxygen is involved in GSSG formation, as was observed for kidney preparation (206, 210, 215). A different result was seen in plasma, with the same glutathione oxidation rate irrespective of the presence of a nitrogen or air atmosphere (207).

In the liver homogenate used in the present study, the total glutathione level remained constant throughout the incubation, with a quantitative oxidation of GSH to GSSG. In plasma, only part of the GSH is known to be oxidised to GSSG, the remainder possibly being converted to a mixed disulphide (207).

Denaturing liver by heating caused only minimal glutathione oxidation during the heating process. The heat-denatured liver preparations incubated in Earl's buffer at

$37^{o}C$ showed a significantly reduced glutathione oxidation rate, when compared with control liver preparations under the same conditions (figure 48). This suggests that oxidation is not solely a chemical effect, but involves enzymic activity. The inhibition of glutathione oxidation on incubation on ice (figure 48) does not discriminate between a chemical or an enzyme mediated mechanism. A combination of both chemical and enzyme effects may well be responsible for glutathione oxidation in the liver, as was observed in the kidney (210), and explains the inhibition of GSH oxidation by heating the liver, and also by addition of EDTA. A low rate of glutathione oxidation has been observed in heat-denatured kidney cells, similar to that in control medium (206). As glutathione oxidation occurs in liver homogenate, this indicates that the effector enzyme does not require an intact cell for activity. The same observation has been made of γ-glutamyl transpeptidase in disrupted kidney cell preparations (206).

Another possible mechanism of glutathione oxidation in tissues is GSH utilization in the reduction of peroxides catalysed by peroxidase (figure 68). Liver homogenate incubated under air, but not under nitrogen, is likely to be under oxidative stress, with detoxification of the hydrogen peroxide produced by both catalase and glutathione peroxidase. Therefore inhibition of catalase by sodium azide should lead to increased utilization and oxidation of GSH. No effect was seen in this study with an azide concentration of 1mM, although a minor effect could have been masked by the glutathione reducing activity of glutathione reductase. In kidney plasma

membrane fraction, sodium azide at a concentration of 50 μM was shown to inhibit catalase without increasing glutathione oxidase activity (215).

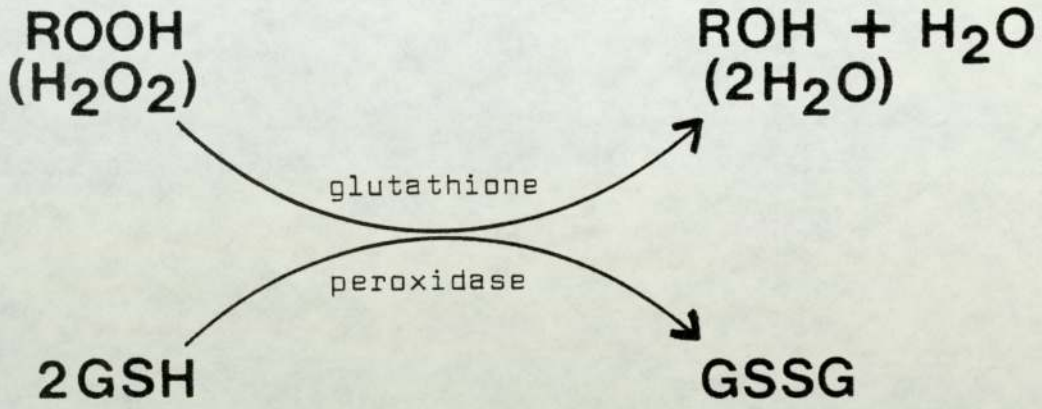


Figure 68 Glutathione oxidation by glutathione peroxidase

In summary, the results presented in section D.4.4 suggest that glutathione oxidation in the mouse liver homogenate preparation requires an enzyme, metal cations and molecular oxygen. If this is so, the decrease in the glutathione oxidation rate caused by monoalkyltriazenes may be due to inhibition of this enzyme or ion chelation. It is puzzling that CyMMT does not inhibit glutathione oxidation, in contrast to a range of other para-substituted monomethyltriazenes (figure 50). Chelation is a possible mechanism of this oxidation inhibition since triazenido-metal bonds can readily be formed as reviewed by Vaughan and Stevens (126). As in the case of the triazene photodecomposition studies (section D.1.1), it is difficult to relate the inhibition of GSH oxidation observed in liver homogenate with the biological effects of triazenes in vivo.

E.11.3 Influence of AcMMT on the glutathione levels in mouse hepatocytes

The effect of AcMMT on glutathione levels was studied further using mouse hepatocytes, in which triazenes are efficiently metabolised (48) (section D.4.2.2). The total glutathione concentration in the mouse hepatocytes (13.0 nmol/10^6 cells) in this study was lower than levels reported for rat hepatocytes (40-50 nmol/10^6 cells) (216). Mouse hepatocytes appear to be more delicate than rat liver cells, as highly viable cells are more difficult to isolate (217). As is usual, the glutathione content of the mouse hepatocytes was expressed in terms of nmol glutathione per total number of cells, rather than per number of viable cells, as determined by the trypan blue dye exclusion test. The low concentration of glutathione in the mouse hepatocytes could be due to an increased leakage of glutathione from dying cells. As in the present work with mouse hepatocytes, glutathione levels in rat hepatocytes have been shown to decrease with time on incubation in a buffered salt-albumin solution (218). It is possible to keep rat hepatocytes *in vitro* with unchanged or even increased glutathione levels by addition of glutathione precursors to the medium (218).

Incubation of AcMMT with mouse hepatocytes for up to 80 minutes resulted in a significant decrease in cellular total glutathione (GSH + GSSG) levels at triazene concentrations of 200 and 500 μM (figures 45, 46), as compared with untreated cells. AcMMT concentrations up to 500 μM were non-toxic as determined by the dye exclusion test, and therefore the decreased total glutathione levels in hepatocytes

were not a result of an increased permeability of the membranes of dying hepatocytes to glutathione. It is not possible to be certain whether the glutathione depletion was either a result of AcMMT metabolism, or a chemical effect as observed with the standard solution of GSH (table 25). However, as glutathione depletion was not seen in liver homogenate after addition of AcMMT, it is proposed that in hepatocytes, glutathione depletion is as a result of AcMMT metabolism, a biotransformation which does not occur in liver homogenate preparations.

AcMMT was able to cause glutathione oxidation of a standard GSH solution in 10% metaphosphoric acid (figure 38). No increase in GSSG levels (as a percentage of total glutathione) was seen in either control or AcMMT treated hepatocytes, which suggests that in the hepatocyte, AcMMT is exerting its effect by a mechanism different from purely chemical interaction. It must be considered that any GSSG formed on oxidation of GSH immediately effluxes from the cells. The total glutathione levels (GSH + GSSG) in the supernatant after spinning hepatocytes down increased slightly with time in both control and experimental incubates, but results were variable (table 26). Glutathione levels in the supernatant could not fully account for intracellular losses, and in triazene treated cells this may be hypothesized to be a result of interaction between glutathione and AcMMT or AcMMT metabolites in the intact cell.

Many other drugs have been reported to cause glutathione depletion in liver. BCNU is a substrate for glutathione-S-transferase in mouse liver cytosol resulting

in production of a BCNU/glutathione conjugate (219). Hydrolysis or metabolism of the substrate with formation of a reactive species is usually the initial step in conjugation with glutathione. For example, a glutathione conjugate is formed of the N-hydroxymethyl species produced on metabolism of N-methyl-4-aminoazobenzene (MAB) (220). Although this type of glutathione conjugate has hitherto only been found in the case of MAB, it is conceivable that similar glutathione conjugates may be formed with the hydroxymethyl derivatives yielded during metabolism of an aryldimethyltriazene (figure 69). Conjugation with glutathione is normally a detoxification mechanism, with hepatoxicity developing only when glutathione levels have been depleted, as for example in the case of bromobenzene (221,222), chloroform (223) and paracetamol (224).

Another mechanism for glutathione depletion was observed by Jones and co-workers (225), studying formaldehyde yielding metabolic substrates in hepatocytes prepared from phenobarbitone pretreated rats. There was no net loss of free glutathione, as would occur with glutathione conjugation; an increase in GSSG concentration in the medium was seen concurrent with glutathione depletion of hepatocytes. Aminopyrine, at a concentration of 5mM, was able to deplete hepatocyte glutathione levels by only 30% after incubation for one hour (225); it therefore seems unlikely that a concentration of 500 μM AcMMT would have a significant depleting effect mediated by formaldehyde alone. Also, in the present study, increased extracellular glutathione concentrations could not account for the fall in intracellular levels.

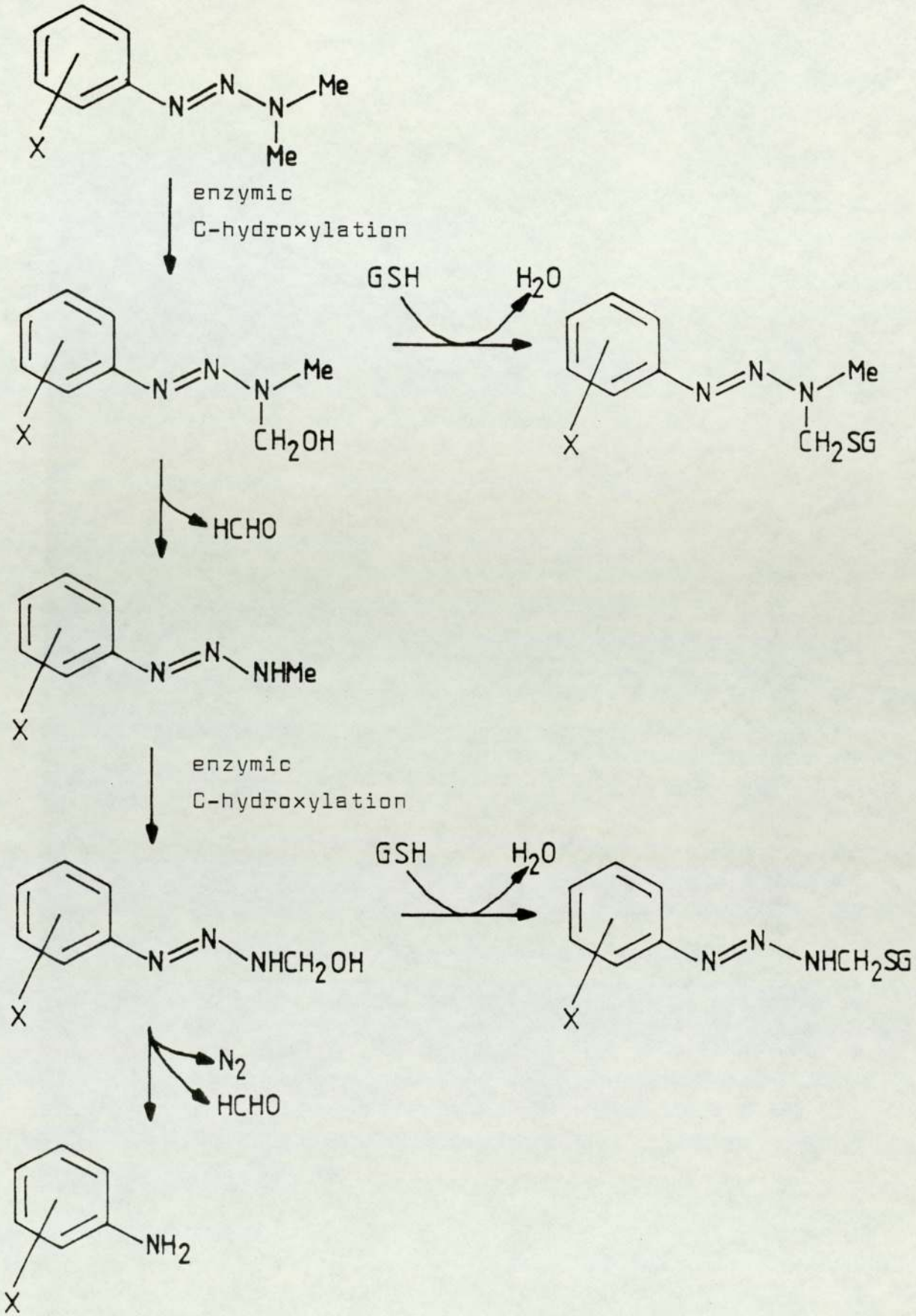


Figure 69 Possible metabolic pathway for an aryldimethyltriazene with formation of glutathione conjugates.

The exact mechanism of depletion as described by Jones is unknown. One proposal was that the formic acid produced on oxidation of formaldehyde by formaldehyde dehydrogenase is able to inhibit the enzyme catalase (225). Under these conditions, hydrogen peroxide produced during NADPH oxidation in cell systems is detoxified by glutathione peroxidase, resulting in glutathione oxidation (225) (figure 70). However, further work demonstrated that there was no direct involvement of free hydrogen peroxide (226) and that some substrates which are not metabolised to produce formaldehyde were also capable of causing GSSG efflux in perfused rat liver (226). Another suggestion was that the utilization of NADPH during metabolic oxidation prevents reduction of oxidised glutathione by glutathione reductase (227), an enzyme which requires NADPH as cofactor. It was later demonstrated however, that addition of effective glutathione depleting substrates only moderately decreased the NADPH concentration in hepatocytes (204), if at all (228). In addition, it was shown that a decrease in NADPH/$NADP^+$ ratio did not necessarily result in GSSG efflux from liver cells (226).

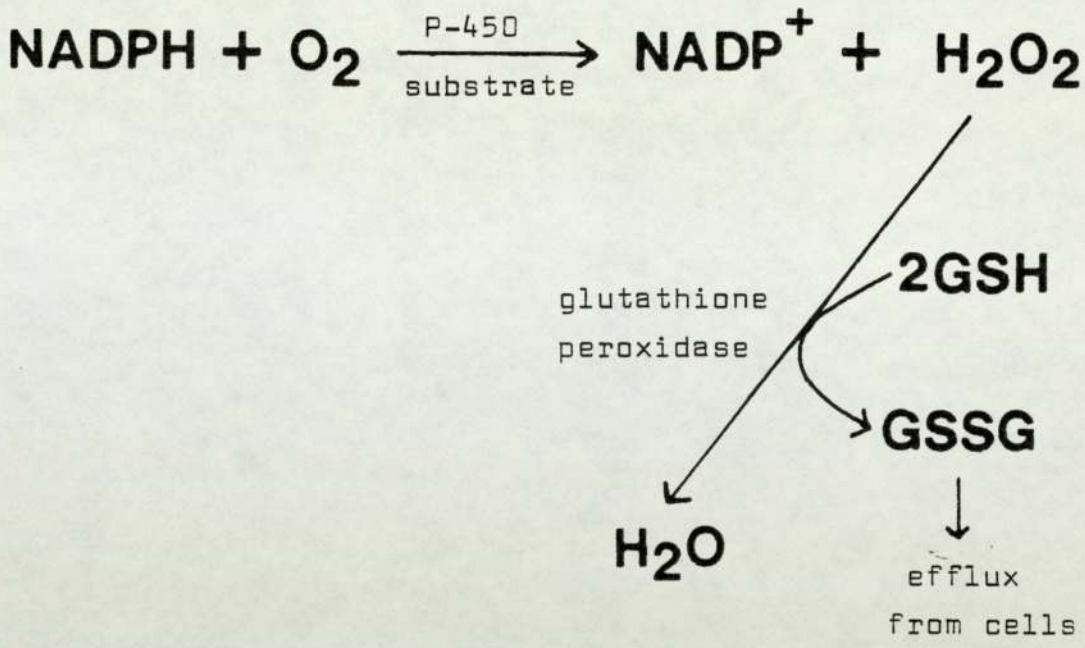


Figure 70 Glutathione oxidation as a result of drug metabolism.

Whatever the exact mechanism of glutathione depletion, it most probably involves changes occuring during substrate metabolism by the cytochrome P-450 system. Dimethyl- and monomethyl- triazenes are metabolised by this system to produce formaldehyde and in theory should be able to cause glutathione depletion from hepatocytes as was described for aminopyrine. In practice, the toxicity studies of AcDMT and AcMMT (section D.3.1) showed that triazenes render hepatocytes non-viable at concentrations above 500 μM and it is unlikely that at the non-toxic concentrations of AcMMT used here, the amount of formaldehyde or hydrogen peroxide formed would be large enough to cause glutathione depletion as observed with 5 mM aminopyrine by Jones et al. Phenobarbitone pretreatment of the animals from which the hepatocytes are prepared (as in the majority

of the work cited in this section of the discussion) may be a prerequisite for observing marked glutathione depletion, since not only does pretreatment cause an increase in the rate of drug metabolism, it also increased hepatic glutathione levels in the rat by 20 - 30% (229), thus making any depletion more easily detectable.

It is impossible to say whether depletion of glutathione is of importance in the mode of action or toxicity of triazenes. *In vivo* studies in mice, using an assay of total thiols by Ellman's reagent, showed that at time intervals after i.p. administration of several triazene derivatives, liver non-protein thiol levels were not reduced by more than 20% of controls (230).

E.11.4 The effect of triazenes on the activity of glutathione reductase in mouse hepatocytes

It is the reduced form of glutathione (GSH) that is used in cells for detoxification reactions, and when utilized with glutathione peroxidase this results in generation of the oxidised form (GSSG) (figure 71). *In vivo*, glutathione is kept primarily in the reduced state by the action of glutathione reductase, which in conjunction with the peroxidase forms the glutathione redox cycle (figure 71). It has been demonstrated that even a very low level of glutathione in hepatocytes is able to detoxify reactive oxygen species if glutathione reductase is functioning optimally (231).

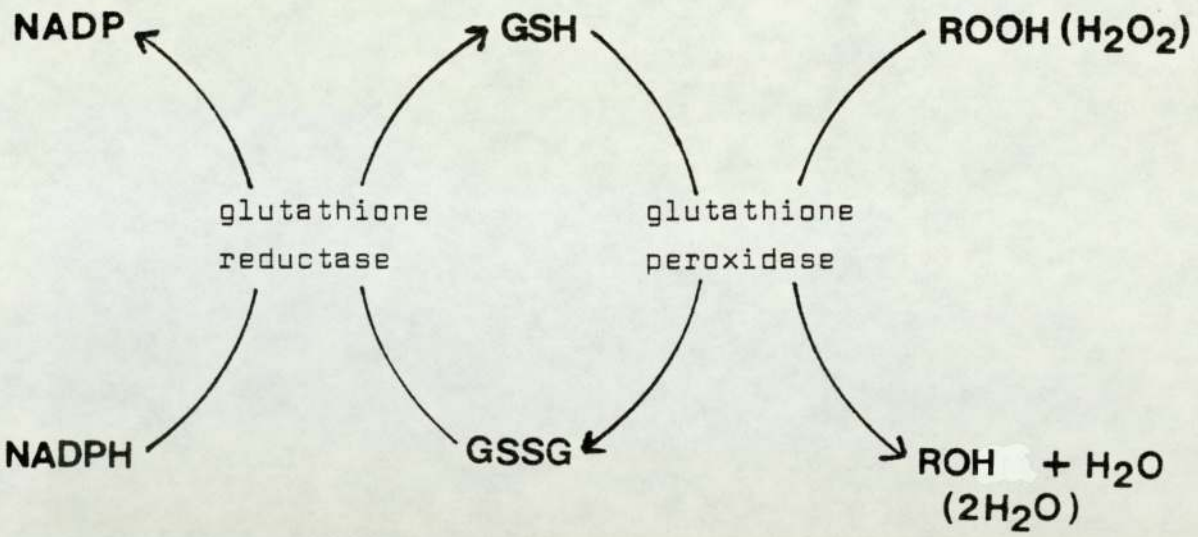
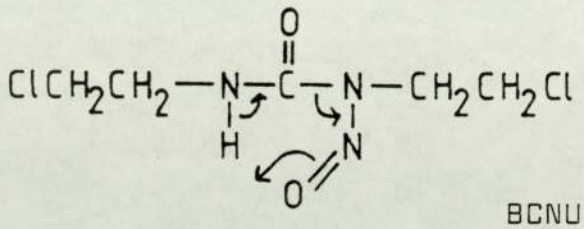


Figure 71 Glutathione redox cycle

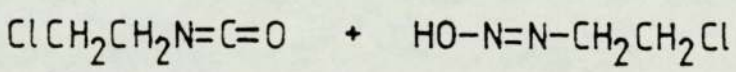
AcDMT and AcMMT were tested as inhibitors of glutathione reductase in mouse hepatocytes (section D.4.3). The hepatocyte was chosen as enzyme source since it is possible that metabolism of the triazene yields an active species which could inhibit an active site of the enzyme. A very slight inhibition ($\sim$ 20%) was demonstrated on incubation of the triazenes with the hepatocytes for 80 minutes (figure 47), but as such a high concentration (500 μM) that any specific interaction is unlikely. Chlorambucil (232) and anthracycline antibiotics (253) have also been found unable to inhibit glutathione reductase.

In a control experiment, BCNU, reported to be a highly specific inhibitor of glutathione reductase in rat hepatocytes (231) was incubated with mouse hepatocytes for 80 minutes. It was able to produce a 65% inhibition of the reductase at a concentration of 50 μM (figure 47). Frischer and Ahmad first demonstrated inhibition of glutathione

reductase activity in the erythrocytes of patients receiving BCNU, with no effect on the activity of other erythrocyte enzymes (234). The inhibition has been shown to be caused by 2-chloroethylisocyanate, a carbamoylating intermediate yielded on decomposition of BCNU under physiological conditions (143) (figure 72).



BCNU



2-chloroethyl-isocyanate + 2-chloroethyl diazene hydroxide

Figure 72 Decomposition pathway of BCNU

Glutathione reductase has been found to be important in the regulation of the biosynthetic activity of melanocytes leading to pigmentation of guinea-pig skin (235). Inhibition of the reductase cannot be responsible for triazene antitumour activity against malignant melanoma, unless DTIC (a cytotoxic agent active against melanoma) is able to cause enzyme inactivation. The cross-resistance

between triazenes and BCNU against TLX5 lymphoma (53) can also not be due to a mechanism involving the inhibition of glutathione reductase. For BCNU, the reductase inactivation seems more closely linked to its toxicity, such as myelosuppression, rather than its antitumour activity (143).

E.12 Conclusion - the identity of the species responsible for triazene antitumour activity

The search for the identity of the active species responsible for dimethyltriazene antitumour activity has continued for over 20 years. Since aryldimethyltriazenes are relatively stable (section D.1.3) and not cytotoxic at physiological pH (section D.3.2), it is now accepted that they require metabolic activation for antitumour activity.

Both monomethyltriazenes and, more recently, hydroxymethyltriazenes have been proposed as candidates for the rôle of selectively cytotoxic species. Monomethyltriazenes, but not hydroxymethyltriazenes would appear to have sufficient stability to circulate *in vivo* to reach a distant tumour target (section D.1.4). There is some evidence that not all N-methyl compounds are demethylated in an identical fashion (section D.2.2) and therefore the formaldehyde precursors produced may possess different properties. Thus there remains the possibility that formaldehyde can be liberated from hydroxymethyltriazenes in a way that results in a selective antitumour effect.

The TLX5 lymphoma has been widely used in the investigation of triazene structure-activity relationships and of the mode of action of antitumour triazenes. It is probably not an ideal tumour model because of its high responsiveness to cytotoxic as distinct from antitumour species, a property it shares with other transplantable murine tumours. On the basis of studies using the TLX5 tumour, the hypothesis has been forwarded that antitumour activity occurs only when a monomethyltriazene can be

produced on metabolism of a dialkyltriazene (45). When dialkyltriazene metabolism was studied in closed tubes under conditions comparable to those in the bioassay where selective cytotoxicity of a dimethyltriazene (Ix) has been observed (47), only products of oxidative N-dealkylation could be identified, and, in agreement with the hypothesis, monomethyltriazenes were metabolically produced in the case of the active antitumour triazene derivatives (section D.2.3).

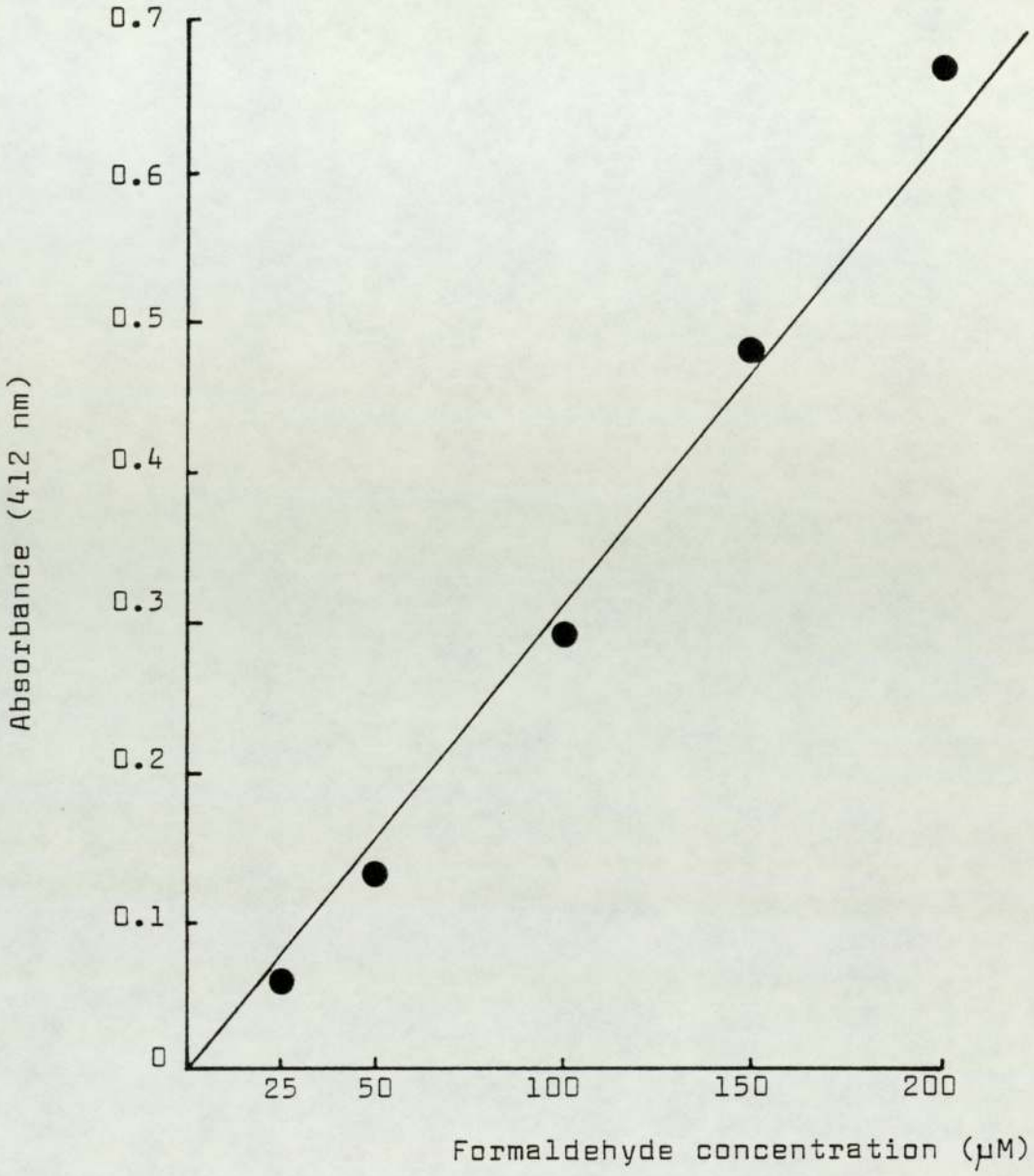
One piece of evidence which contradicts the hypothesis that monomethyltriazenes are the selectively active triazene species is the finding that a monomethyltriazene (Iy) was equitoxic to both triazene sensitive and resistant TLX5 cell lines in a bioassay (47). However, the results obtained *in vitro* as part of the present work do not agree with those of the bioassay studies, since preferential cytotoxicity of a monomethyltriazene (AcMMT) was seen against the triazene sensitive TLX5 line, as determined by the trypan blue dye exclusion test (section D.3.2). These results and those of an *in vivo* study using the TLX5 lymphoma (47) suggest that the monomethyltriazene may be responsible for the selective cytotoxicity of dimethyltriazenes. It would be interesting to conduct further *in vitro* studies where TLX5 and TLX5R cells are incubated with a monomethyltriazene at the same concentrations as used in the bioassay, with the aim of obtaining cell kill by the same mechanism as in the bioassay experiments. After two hours, the cells should be washed and grown *in vitro* until cell numbers are great enough for counting, rather than implanted in mice to grow *in vivo*, as is the standard procedure in the bioassay (47).

If the monomethyltriazenes are indeed the active antitumour species, an explanation must be found for the observation that monoethyltriazenes are not active compounds *in vivo* (45), although just as potent alkylating species *in vitro* (92). In the present study, it was shown that both monomethyl- and monoethyl- triazenes are metabolised at the same rate (section D.2.4), hence both species should be equally bioavailable as alkylating agents. There is of course a possibility that *in vivo* metabolism of the monomethyltriazenes, but not the monoethyltriazenes, yields a species that is also an active antitumour compound, even though results of *in vitro* studies do not support this contention (section E.7). Whereas it is quite feasible to assume that monomethyltriazenes exert their carcinogenic effects by indiscriminate alkylation, it is much less clear whether their antitumour properties are a result of alkylation of DNA. In fact there are reports of equivalent labelling of nucleic acids in triazene sensitive and resistant human malignant melanoma cells upon incubation with DTIC (61). Any other site of action, irrespective of whether alkylation is involved, may not be responsive to the slightly different properties of the monoethyltriazenes. Further investigations which could produce new leads include alternative sites of action for example crucial enzyme systems such as those involved in energy production or glutathione metabolism and utilization, and comparisons of the biochemical properties of triazene sensitive and resistant cell lines.

In the present work, there was one result which suggests that the monomethyltriazene was not responsible for the cytotoxicity of the dimethyltriazene (section D.3.1), since, in hepatocytes the monomethyltriazene, AcMMT, appeared to be less cytotoxic than the dimethyltriazene, AcDMT. However, in this study, it is not known if the cytotoxicity produced in hepatocytes is equivalent to that in the TLX5 tumour cells.

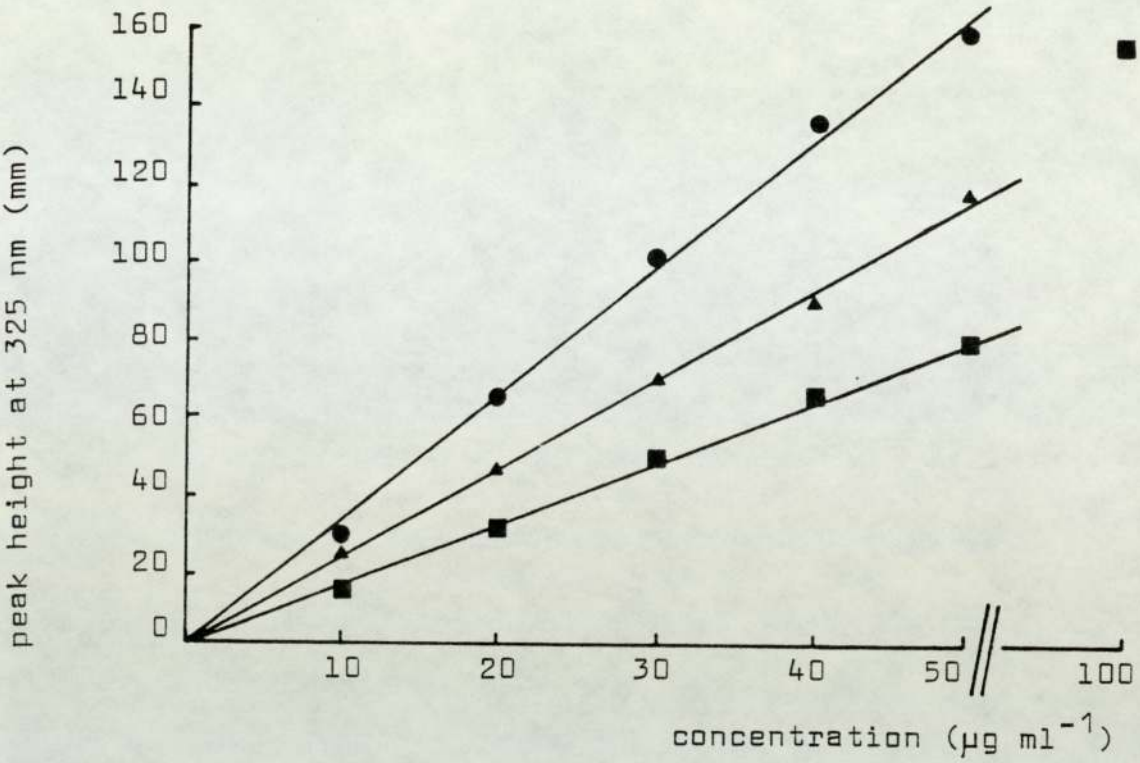
In conclusion, it seems, on balance, probable that the monomethyltriazenes are responsible for triazene anti-tumour effects, but the mode of action is still unclear, and possibly involves a mechanism other than alkylation of DNA.

APPENDIX

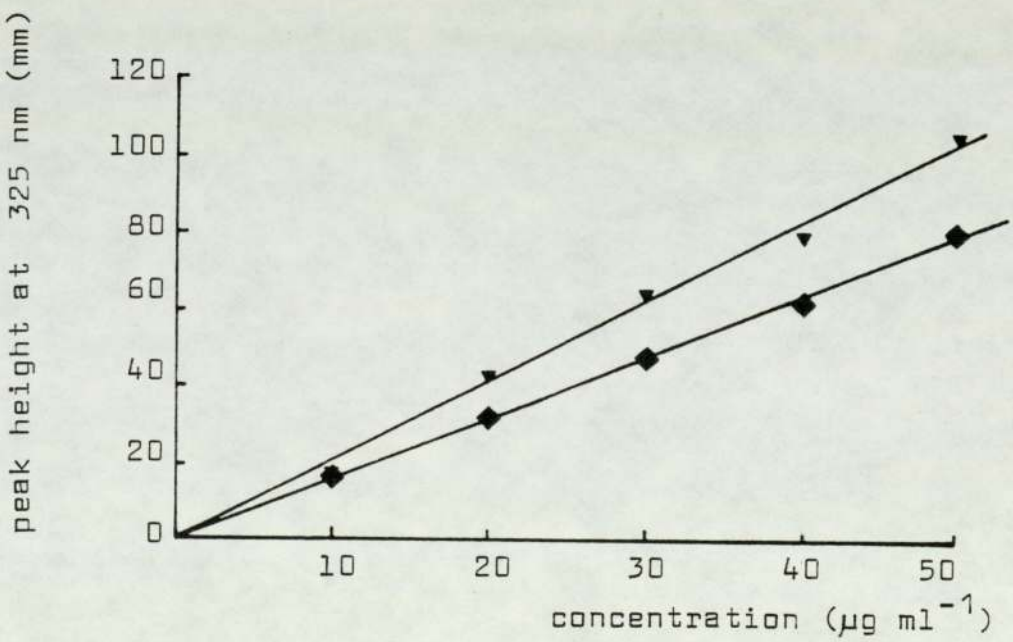


Formaldehyde calibration curve

correlation coefficient = 0.999



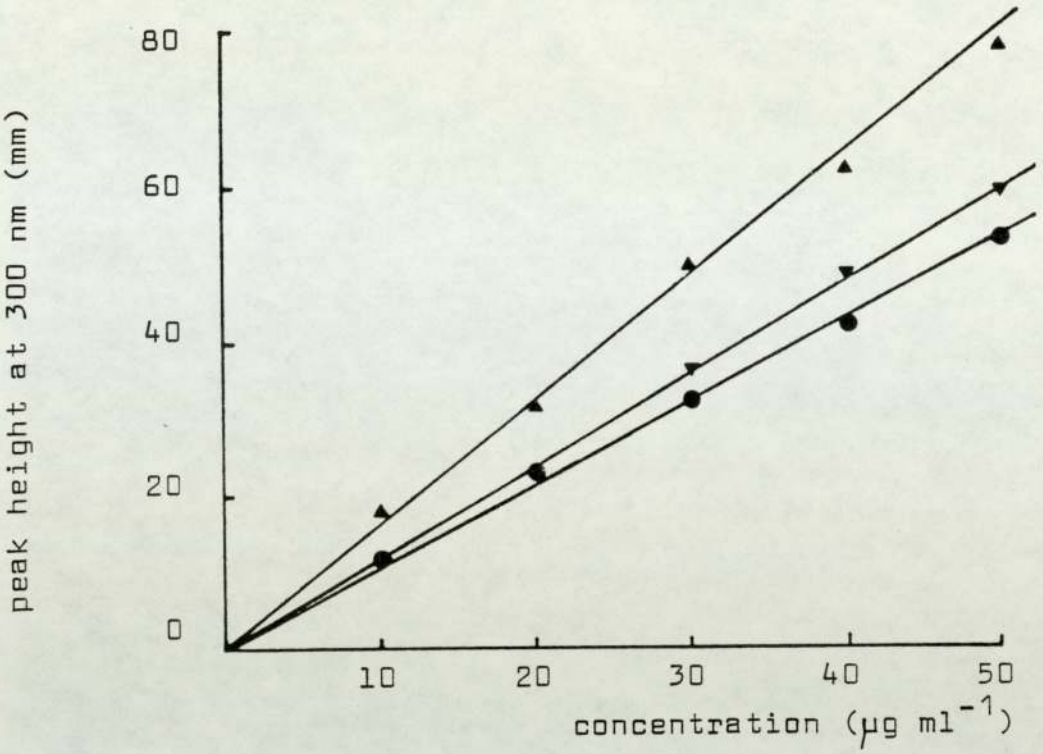
Calibration curves for metabolites of AcDMT (●) - (Xa), (▲) - AcMMT, (■) - (Io).



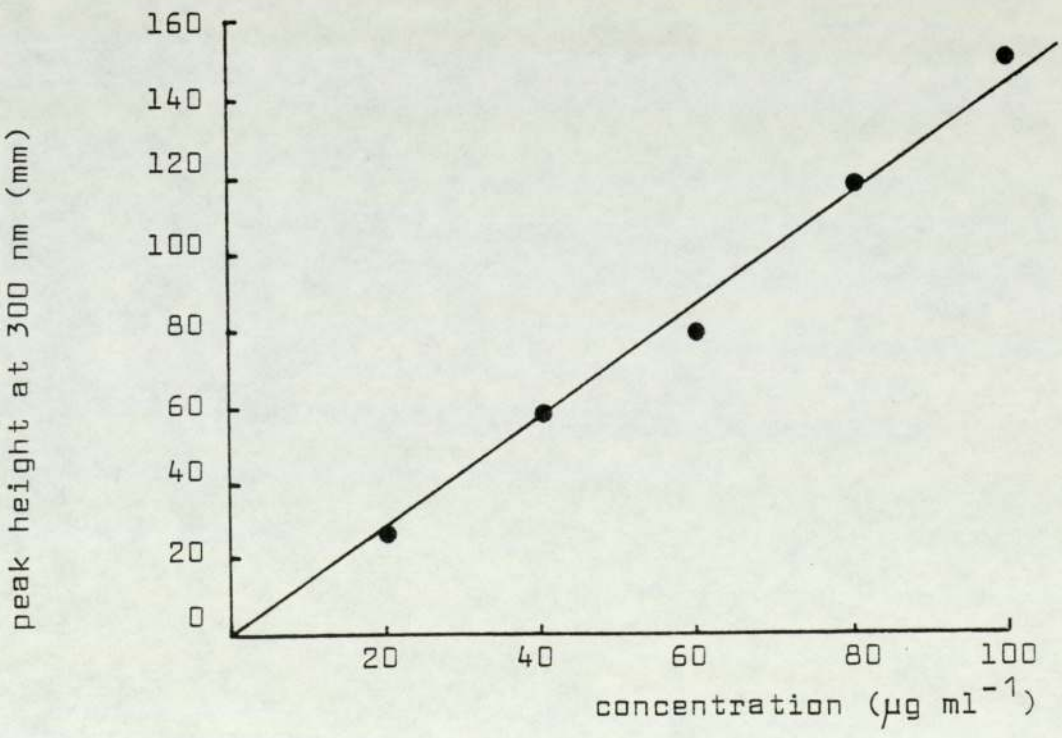
Calibration curves for metabolites of AcDET (▼) - AcMET, (◆) - (Ip)

Detector sensitivity = 0.32

For HPLC conditions, see table 5



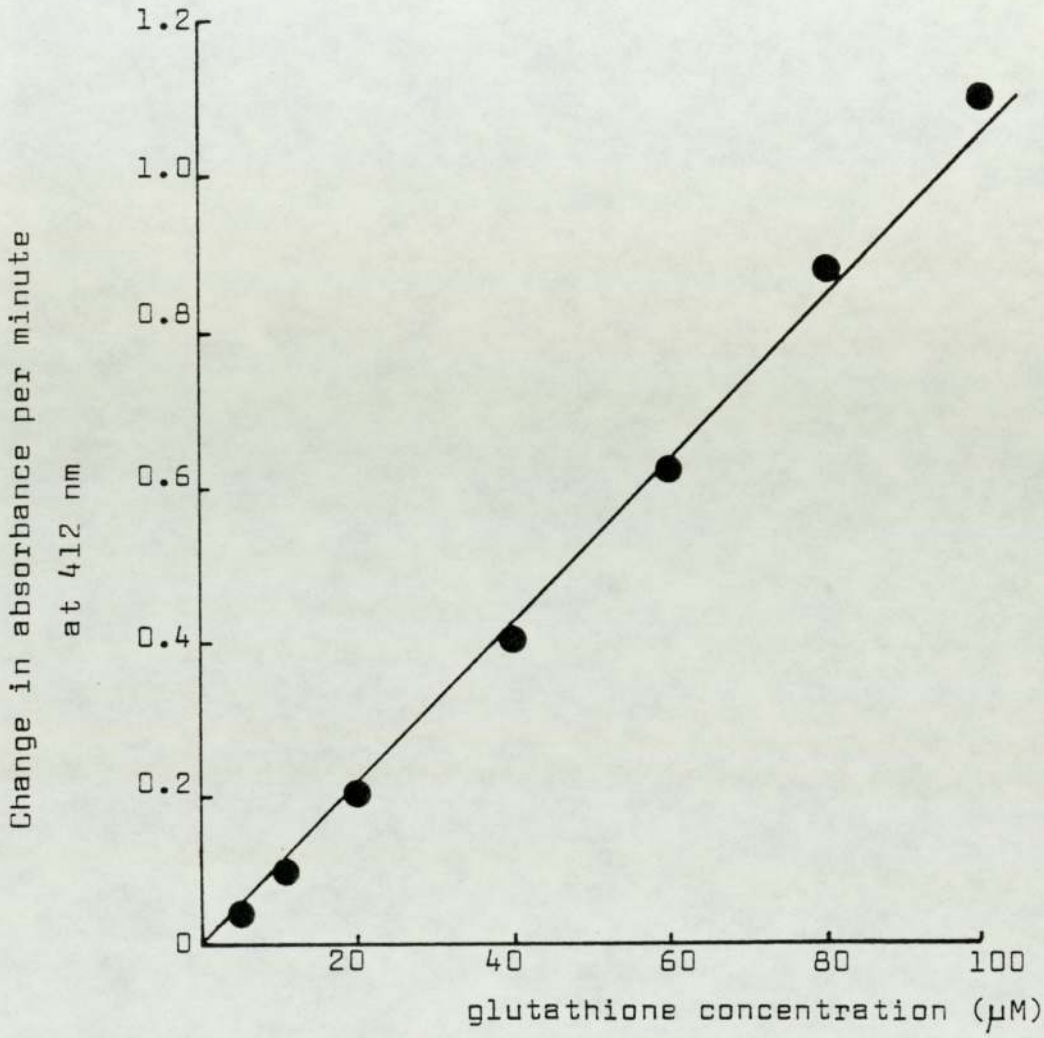
Calibration curves for metabolites of CyDMT, CyDET and (Iu). (▲) - CyMMT, (▼) - CyMET, (●) - (Xb)



Calibration curve for metabolite of (Iv) - (Xb)

Detector sensitivity = 0.32

For HPLC conditions, see table 5



Glutathione calibration curve

Glutathione concentrations are expressed in GSH equivalents (GSH + $\frac{1}{2}$ GSSG)

correlation coefficient = 0.999

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